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THE CAROTENE CONTENT OF MARKET HAYS AND CORN SILAGE[1]

By HERBERT G. WISEMAN, *assistant chemist*, EDWARD A. KANE, *assistant chemist*, LEO A. SHINN, *assistant chemist*, and C. A. CARY, *senior chemist, Division of Nutrition and Physiology, Bureau of Dairy Industry, United States Department of Agriculture*[2]

INTRODUCTION

The importance of vitamin A in cattle rations has been recognized for several years; and as more definite knowledge has accumulated regarding the quantity of vitamin A required by dairy cattle, more exact information about the vitamin A potency of farm feeds has become desirable and usable in feeding these animals. Although a number of conditions besides the quantity of vitamin A or its precursors may affect the vitamin A potency of a feed, the determination of the quantity of its precursors in feeds of plant origin is, at the present time, by far the best available measure of their vitamin A value.

In connection with the work done in the laboratory of the Division of Nutrition and Physiology at Beltsville, Md., to determine the vitamin A requirements of cattle, all the market hays that have been fed and many samples of the corn silage fed, have been analyzed for carotene. In addition, the hays have been graded by representatives of the Division of Hay, Feed, and Seed of the Bureau of Agricultural Economics.

There have thus been collected considerable data on the carotene content of the various market grades of hay—particularly of alfalfa and timothy hays—and the relation between certain physical characteristics of these hays and their carotene content, which might be of use in determining to what extent hays of suitable vitamin A potency may be selected by ordinary inspection or grading.

In analyzing the samples of corn silage, it was early noted that corn silages differ greatly in their vitamin A value and that while some are a rich source of vitamin A others contain too little to be used as the principal source of vitamin A in cattle rations. Work was therefore done to determine some of the conditions that influence the vitamin A value of corn silage.

The routine analytical methods that have been used in the Beltsville laboratory, and also in other laboratories, for the determination of carotene in cattle feeds have depended upon some modification of the well-known Willstätter and Stoll procedure for separating chlorophyll and xanthophyll from carotene. More information is needed regarding the accuracy of these methods when applied to hays and silages,

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and possibly also when they are used in analyzing the freshly cut green plant. Considerable work has also been done in various laboratories by using modifications of the Willstätter and Stoll procedure to separate the cholorophyll and xanthophyll from the carotene, and it seemed desirable to know to what extent the results from these different laboratories are actually comparable.

In this paper, the writers (1) describe the procedure used at present in the Beltsville laboratory to determine the carotene content of cattle feeds; (2) consider the results they have obtained on the accuracy of this procedure and the comparability of such methods when applied to hays and corn silage; and (3) present the data they have obtained on the carotene content of market hays and corn silage, together with a consideration of the practical usefulness of these results in feeding dairy cattle. These topics are taken up practically in the order mentioned; but, before proceeding with this outline, the writers discuss briefly some of the more closely related investigations that have led up to some phases of the work reported on in this paper. No attempt is made, however, to review all the literature on the vitamin A value of roughages, much of which is doubtless familiar to most workers who are interested in the subject.

REVIEW OF PREVIOUS, RELATED INVESTIGATIONS

It has been 30 years since work was begun in Wisconsin on cattle that were observed to be suffering from symptoms that are now known to result (*10*, *25*),[3] in part at least, from a deficiency of vitamin A in the ration. This factor had not been distinguished as a separate dietary essential; and a long period intervened before cod-liver oil was found to ameliorate these symptoms in cattle (*10*), and before it was shown that for the normal nutrition of calves the diet must contain some source of vitamin A (*14*). It is now well known (*2*, *3*, *11*, *27*, *28*, *29*) that vitamin A or its precursors may be so deficient in ordinary cattle rations as to lead to disaster in reproduction, to difficulty in rearing the young, or to a deficiency of vitamin A in the milk. Also, several laboratories (*9*, *29*, *32*) have reported work on the quantity of vitamin A required by cattle to bring about certain physiological responses.

Formerly, alfalfa hay was generally regarded as an excellent source of vitamin A; but Meigs and Converse (*27*, *28*, *29*) found that the failure in reproduction—as well as the deficiency of vitamin A in the milk—which they observed when the cows were fed a poor quality of alfalfa hay continuously as the main source of vitamin A, was due to the low vitamin A value of the ration they were feeding. They also encountered similar results when the poorer grades of timothy hay were similarly fed.

Previous work in the Beltsville laboratory (*12*) with rats had shown that the carotene in alfalfa hay accounts completely for its vitamin A potency. Under suitable conditions, all the known precursors of vitamin A in plant materials occur in the carotene fraction, when analytical methods based on the Willstätter and Stoll procedure are used for the separation of the plant pigments. So little of these precursors of vitamin A exist in the grains used in dairy rations that, even with a liberal amount of yellow corn in the ration, a cow usually

[3] Italic numbers in parentheses refer to Literature Cited, p. 667.

must depend on the roughages in the ration for well over 90 percent of her vitamin A requirements.

Beta-carotene is by far the most important precursor of vitamin A in hays and corn silage. It appears to be practically the only one in alfalfa (*41*) and the grasses (*15, 22, 31*). Little is known regarding the relative quantities of this pigment that cattle assimilate from various plant sources and dietary mixtures. Evidence has been presented to show that soybeans, for instance, may possibly affect the utilization of carotene in the ration, at least insofar as the effect of this pigment upon the vitamin A content of butter is concerned (*13*). Nevertheless, it appears that the quantity of beta-carotene in hays and corn silage is by far the best available measure of the relative value of these feeds as a source of vitamin A.

The other known precursors of vitamin A in plant materials are much less efficiently utilized than beta-carotene (*5, 21*); and the amounts in which they occur in most cattle feeds are relatively negligible. The carrot is a notable exception, the carotene from its root being a mixture of about 10 to 20 percent of alpha-carotene, traces of gamma-carotene, and 80 to 90 percent of beta-carotene (*15, 22, 41*). Although cryptoxanthine is said to account for most of the vitamin A potency of the yellow corn kernel, the proportion of the vitamin A in cattle rations that is due to this pigment is quite small. The proportion of the vitamin A potency of corn silage that is due to the cryptoxanthine in the corn kernel is also negligible.

The accuracy of the determination of the vitamin A potency of alfalfa or timothy hay or of corn silage depends, therefore, upon the accuracy with which the quantity of beta-carotene in these feeds can be estimated. In most of the analyses at hand, it has been assumed not only that practically all of this pigment in these feeds occurs in the final carotene fraction that is used to estimate it either colorimetrically or spectrophotometrically, but also that no other pigment that would affect these measurements is present in this carotene fraction. This latter assumption may be very nearly true for such freshly cut, green plant materials as the grasses and alfalfa (*46*); but carotene fractions from a sample of alfalfa hay that were tested biologically for vitamin A potency in the Beltsville laboratory were found to be somewhat less active than the amounts of beta-carotene estimated on the basis of this assumption to be present in them. The difference in the growth of the rats used in this experiment, however, amounted to only about 20 percent (*12*).

Further, it is known that in plants cut for hay, there is a very rapid destruction of the carotene during the curing process (*36, 37*), and that this destruction continues, although at a somewhat slower pace, during subsequent storage (*8, 46*). Intermediate colored decomposition products are known to be formed in the oxidation of carotene (*18, 26*); and, according to Kuhn and Brockmann (*18*), not all of such products are readily removed from the carotenes by methods of analysis based on the Willstätter and Stoll procedure for the removal of the chlorophylls and xanthophylls. One might therefore suspect the presence of such pigmented impurities in the hays even if they did not occur in the freshly cut plant materials.

It is further known that the carotenes to some extent, but more especially the xanthophylls, are attacked by acids. Kuhn (*18*) and

his collaborators found that the xanthophylls thus yield colored decomposition products that may also contaminate carotene fractions prepared by the Willstätter and Stoll procedure (*18*, *23*). It seems, therefore, that the possibility of the occurrence of such xanthophyll decomposition products in the carotene fractions, especially from the silages, should also be considered.

Several biologically active oxidation products of carotene have been prepared (*4*, *19*, *20*); but the only naturally occurring products of this sort that have been tested biologically for vitamin A activity, apparently, are those that have been tested in the Beltsville laboratory, and these have been found to be inactive.

Kuhn and Brockmann (*18*) describe adsorption methods for the separation of carotene from its colored oxidation products and from the products formed from xanthophyll by the action of acids. No evidence is presented by these workers, however, to show that the carotene itself may not be altered in the procedure.

ANALYTICAL PROCEDURE FOR THE DETERMINATION OF CAROTENE IN FARM FEEDS

DESCRIPTION OF ROUTINE ANALYTICAL METHODS

Two analytical methods (A and B) have been used for the routine determination of carotene in the work reported in this paper. They differ only in the way the samples of feed were ground and the carotene extracted from them. Method B is described here as it is used in analyzing hays and such succulent materials as the silages and the freshly cut plant. Both methods A and B, like most methods in practical use, depend upon a modification of the Willstätter and Stoll procedure for separating the carotene from the other plant pigments.

METHOD A

Method A is usable with materials that may be ground readily and completely in a Wiley mill. It is particularly useful with hays. With baled hays the bales are bored repeatedly with a 2-inch drill. The drillings are ground through the medium sieve (1-mm holes) and then through the finest sieve (0.5-mm holes) in a Wiley mill. Aliquot samples are taken for analysis. Absolute ethanol, 15 ml for a 6-g sample, is added; and, after standing 1 hour or more (overnight), the mixtures are extracted six times or more by shaking for 15 minutes with low-boiling (30° to 60° C.) petroleum ether (20-ml portions for a 6-g sample). A sintered glass funnel is used in removing the extract. The combined extract is concentrated in vacuo to 75 to 100 ml; 20 ml of water is added; and after the solution is shaken and mixed by swirling gently, the alcohol-water layer is discarded. The chlorophyll and about 95 percent of the xanthophyll are removed from the carotene by two treatments of the ligroin fraction with a saturated potassium hydroxide-methanol (10 ml each time) solution. The mixture is shaken 15 minutes during each treatment; the alcoholic layer is then diluted with 1 ml of water, the shaking continued for 2 to 5 minutes more, and the diluted alcoholic potash layer is discarded. The petroleum ether fraction is then washed free of xanthophyll by shaking it for 2 minutes repeatedly with 25-ml portions of methanol, 92 percent by volume. The methanol used in the final washing should be colorless.

The carotene fraction is then concentrated in vacuo and brought to volume with a 1:1 mixture of ethanol and higher boiling (about 90° C.) petroleum ether. The ethanol retards the oxidation of the carotene.

Method B

Method B is suitable for the determination of carotene in all sorts of material; but it has been found especially useful with dry materials that are difficult to grind satisfactorily in a Wiley mill, and for succulent substances such as the freshly cut plant, silages, etc. In this method the sample is ground to a fine powder while immersed in concentrated ethanol (approximately 98 percent by volume after allowance is made for the moisture from the sample).

Dry materials are first ground through the medium sieve (1-mm holes) in a Wiley mill, and then with alcohol in the ball mill.

The corn plant, when analyzed, is first put through a meat grinder and the ground material is collected directly in absolute ethanol. This mixture is allowed to stand overnight in an ice box; the aqueous-alcohol fraction is then removed and the residue ground in a fresh lot of absolute ethanol in the ball mill. The silages were formerly handled in the same way as the freshly cut corn plant; but later it was found that corn silage need not be ground in a meat grinder before it is immersed in the alcohol.

With freshly cut plant materials such as grasses, alfalfa, etc., absolute ethanol is taken into the field and cooled with solid carbon dioxide to between −60° and −70° C. The sample when cut is immediately frozen in this mixture and the brittle material broken up with a pestle. After standing overnight in an ice box, the sample is handled in the same way as the silages.

With succulent materials enough alcohol is used in the preliminary alcoholic treatments to give an alcohol-water mixture containing close to 90 percent or more of alcohol. This alcohol-water mixture is poured off quite completely; a Büchner funnel is used, if necessary, to recover the residue. The second alcoholic extract, which contains almost all the carotene, may be filtered onto a Büchner funnel, the residue being washed thoroughly with additional alcohol; or the residue simply may be allowed to settle out, the water from the original sample being allowed for in calculating the total volume of this extract.

The two alcoholic extracts obtained in these extractions are aliquoted separately and the aliquots combined. Water is added to reduce the alcoholic concentration to 85 percent; the carotene is extracted out of this diluted alcoholic solution by shaking twice with the low-boiling petroleum ether; and the combined carotene extract is then saponified, washed with 92-percent methanol and brought to volume as in method A.

In the ball mill, 1,600 ml of absolute ethanol have been used with 100-g samples of the hays or freshly cut grasses or freshly cut alfalfa and with 300-g samples of corn silage or of the freshly cut corn plant. In the preliminary alcoholic treatments, with the samples from the corn plant and corn silage 3 liters of absolute ethanol were used and 1 liter with the grasses and freshly cut alfalfa. All of the petroleum ether used in this work was treated with concentrated sulphuric acid,

washed with water, and then treated with potassium permanganate to remove peroxides.

All the samples reported in this paper have been read with a spectrophotometer—many at wave length 436 m μ. The carotene has been calculated on the basis of $E^{1\mu g \text{ per ml}}_{1cm}=0.195$ at wave length 436 mμ. Frequently the samples have also been read at other wave lengths to obtain light on the character of the pigmented material in them. A colorimeter equipped with a mercury light and a light filter that transmits the 436 or 436 and 405 bands of light, when used with a potassium dichromate standard, gives relative results that are practically identical with those obtained with the spectrophotometer at wave length 436 mμ. The photoelectric machine described by Brice (*1*) has also been found to be very satisfactory when adequately calibrated and used with a light filter. The Corning Glass Works filter No. 556 has been used.[4] [5]

LOSS OF CAROTENE IN THE GRINDING AND EXTRACTION OF HAYS FOR CAROTENE ANALYSIS

Previous work in the Beltsville laboratory (*12*) has shown that when finely pulverized hay is extracted by method A, no biologically detectable amount of carotene is destroyed, and only a trace of vitamin A potency remains in the extracted residue. In that experiment this trace unquestionably amounted to only a small fraction of 10 percent of the total carotene originally in the powdered hay. The data in table 1 indicate further that it is unlikely that any considerable loss of carotene occurs during the grinding of a hay to a fine powder while it is immersed in alcohol in a ball mill as in method B. The results that are summarized in table 2 show, also, that the extraction of carotene from hays by method B is practically as complete as by method A, and that the method of grinding used in method A, is, therefore, very nearly as satisfactory as that in method B.

TABLE 1.—*Effect of the duration of grinding hay samples, while immersed in alcohol in a ball mill, on the carotene found*

Alfalfa hay sample No.[1]	Carotene per kilogram found when hay sample was ground for—				
	2 hours	3 hours	4 hours	5 hours	17 hours
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
1	53.9	53.9	54.3	54.3	53.4
2	----------	81.0	----------	79.0	79.6

[1] The spectral absorptive properties of the different aliquots of each sample of hay were the same regardless of the length of time of grinding.

[4] The samples were read visually on a Bausch & Lomb Universal spectrophotometer in the color laboratory of the Bureau of Chemistry and Soils at Arlington, Va. Many of the samples were read at Beltsville by either the colorimetric or the photoelectric procedure, described here, before and after they were read at Arlington. There was no apparent loss of carotene in taking the samples to Arlington. Ten or more readings were made at each wave length of light used in this work, the cups being switched.

[5] Some modifications in procedure have been made from time to time, but none of these has affected materially the results included in this paper.

TABLE 2.—*Comparison of results for carotene as obtained by methods A and B*

Samples of hay analyzed (number)	Method of analysis	Average carotene content per kilogram
		Milligrams
Average of 4 samples	Method B	28.8
	Method B, with subsequent extraction of the powdered residue with ligroin as in method A.	29.6
Average of 9 samples	Method A	17.2
	Method A, with subsequent extraction of the powdered residue with ligroin as in method A.	17.4
Average of 6 samples	Method A	15.5
	Method B	16.1

Efforts to obtain additional carotene from hay residues, after they have been ground and the carotene has been extracted according to method A, by treating them again with alcohol and by repeated extractions with ligroin have yielded further traces of pigment which at times may have been equivalent to as much as 3 or 4 percent of the total amount of carotene found in the hay. This small amount of carotene has been disregarded in the results presented in this paper.

The results cited in tables 1 and 2 favor the conclusion that very little, if any, destruction of carotene occurs in the grinding and extraction procedure by either method and that only a negligible amount of this pigment remains in the hay residues after extraction.

When method B is used to determine the amount of carotene in silages and succulent plant materials, the extraction of this pigment is undoubtedly as complete as with the hays, as the final conditions under which the extraction is carried out are the same. Further, in the analysis of freshly cut plant materials which are immediately immersed in alcohol at −60° to −70° C., the destruction of carotene must certainly be very small. Even when the sample is not immediately frozen in alcohol but is simply ground through a meat grinder, collected in the alcohol at room temperature, and analyzed as described in method B, the loss of carotene is still negligible. For example, when three lots of freshly cut, green alfalfa were analyzed by these two methods, the average carotene content per kilogram of dry weight was 239 and 214 mg, respectively. It would appear, therefore, that insofar as the grinding and extraction procedure is concerned, method B is as satisfactory for determining the carotene content of freshly cut plant materials and silages as it is for hays.

LOSS OF CAROTENE IN THE SAPONIFICATION OF THE CHLOROPHYLL AND IN THE SUBSEQUENT WASHING OF THE LIGROIN SOLUTION WITH METHANOL, AND THE COMPLETENESS OF REMOVAL OF THE XANTHOPHYLL AND CHLOROPHYLL

The data in table 3 show that when a solution of pure beta-carotene in low-boiling ligroin is treated with a saturated solution of potassium hydroxide in methanol to remove the chlorophyll, as described in method A, the loss of carotene in the two saponifications that are necessary for this purpose is less than 1 percent. There was no evidence that the carotene was altered chemically by these treatments. Along with the chlorophyll, about 93 percent of the xanthophyll was also separated from the carotene.

TABLE 3.—*The loss of beta-carotene and the removal of leaf xanthophyll from the carotene when solutions of these pigments in low-boiling petroleum ether are treated with a saturated solution of potassium hydroxide in methanol for the removal of chlorophyll from carotene as described in method A*

Pigment originally in ligroin solution	Quantity initially present	Initial volume	Saponification treatments	Dilution of alcohol before separation	Quantity finally in petroleum ether layer	Removed from petroleum ether layer
	Micrograms	*Milliliters*	*Number*	*Percent*	*Micrograms*	*Percent*
Beta-carotene	63.15	[1] 50	[2] 8	92	63.10	0.1
				90	62.18	1.5
Xanthophyll	1,280.00	[3] 100	2	92	84.00	93.4
				92	78.00	93.9

[1] The volume was made to 50 ml at the beginning of each saponification.
[2] The spectral absorption of the initial and final carotene solutions was determined at wave lengths 430 mμ, 450 mμ, and 480 mμ. The relation between the absorptions at these wave lengths was not changed as a result of these 8 saponifications.
[3] These solutions were saturated. The leaf xanthophyll that was used melted at 177° to 178° C. (uncorrected).

The results in table 4, with leaf xanthophyll in low-boiling ligroin, indicate that over 99 percent of it is removed from this solution by five washings with 92-percent methanol as described in method A. The trace of pigment that appeared to remain in the petroleum ether—possibly about 0.5 microgram per milliliter [6]—was not removed by further washing. The quantity of xanthophyll in these samples was approximately four times as much as could be present in a carotene analysis after removal of the chlorophyll as described above. The results show a more complete extraction of the xanthophyll by the 92-percent methanol than was observed by Miller (*30*).

TABLE 4.—*Completeness of removal of xanthophyll from its solution in low-boiling petroleum ether by washing this solution with 92-percent methanol as described in method B* [1]

Sample No.	Xanthophyll initially in sample	Volume of petroleum ether layer		Washings	Xanthophyll finally in petroleum ether solution	Xanthophyll concentration per milliliter in final volume	Xanthophyll removed
		Initial	Final				
	Micrograms	*Milliliters*	*Milliliters*	*Number*	*Micrograms*	*Micrograms*	*Percent*
1	360	75	58	5	3.0	0.05	99.2
		75	54	6	3.1	.06	99.1
2	360	75	54	5	2.7	.05	99.2
		75	49	6	2.5	.05	99.3

[1] The final extract was colorless in all cases.

The data in table 5 show the amount of pure carotene that may be lost from the ligroin phase as a result of washing its solution in low-boiling ligroin with diluted methanol to remove the xanthophyll. The loss with 90-percent methanol and 10 washings or with 92-percent methanol and 5 or 6 washings, is slightly over 1 percent.

[6] This was estimated photoelectrically from a very slight absorption of light by this solution.

TABLE 5.—*Loss of beta-carotene as a result of washing its solution in low-boiling ligroin with diluted methanol for the removal of the xanthophyll as described in method A*

Sample No.	Pigments initially in ligroin layer		Initial volume of ligroin layer	Washings	Temperature	Concentration of methanol	Pigment calculated as carotene left in ligroin layer	Recovery of carotene, based on carotene in original solution
	Kind [1]	Quantity						
		Micrograms	*Milliliters*	*Number*	*°C.*	*Percent*	*Micrograms*	*Percent*
K-1, I-8	Beta-carotene	52.50	25	[2] 10		92	[3] 51.30	[3] 97.7
K-2, I-8	do	52.50	25	[2] 10		90	[3] 52.00	[3] 99.0
W-1, I-8	do	63.15	[4] 50	[2] 10		90	62.38	98.8
W-2 and 3	do	575.00	100	5	28	92	572.50	99.6
W-4 and 5	do	575.00	100	5	15–17	92	568.00	98.8
W-6 and 7	do	531.00	100	6		92	522.00	98.3
W-8 and 9	do	531.00	100	6		92	527.00	[5] 99.2
	Leaf xanthophyll	1,045.00						

[1] The melting point of the leaf xanthophyll was 177° to 178° C. (uncorrected). The beta-carotene extracts in the I-8 samples were fractions of the second international standard carotene.

[2] The initial and final solutions were read spectrophotometrically (visual method) at wave length 450 mμ, 480 mμ, and at either 430 mμ or 436 mμ. There was no evidence of any change in the character of the pigment as a result of these washings.

[3] There was some mechanical loss with this sample. The loss was larger with K-1 than with K-2, but an examination of the methanol layer by driving the ligroin dissolved in it out of solution by the addition of water showed some color in this layer in the case of the 92-percent methanol.

[4] This sample was made to 50 ml before each extraction.

[5] The final volume of the ligroin solution was 80 ml with each sample. If the concentration of the xanthophyll left in the ligroin phase is the same as shown in table 3, the unextracted xanthophyll would be 4+ μg, and the recovery of the carotene corrected for this xanthophyll would be 98.5 percent.

The results in table 6 indicate the extent of the loss of the carotene that may occur in the routine analysis of alfalfa hay when the procedure described in method A is used to remove the chlorophyll and xanthophyll. The loss in washing with 92-percent methanol is not detectably greater than it was with pure beta-carotene itself; but in saponifying to remove the chlorophyll this loss is apparently somewhat greater—being about 2 percent for two treatments of this nature. In another effort to get at the magnitude of these losses the final carotene fraction from a sample of hay was divided into two aliquots. One was saponified four additional times as described in method A and then washed seven additional times with 92-percent methanol. The decrease in absorption that could be attributed to a loss of carotene was between 1.5 and 3.4 percent; and, as will be shown presently, these "carotene" fractions from hays and silages contain pigments other than carotene that may account for a considerable portion of this loss.

TABLE 6.—*Loss of carotene from alfalfa hay in separating the carotene from chlorophyll and xanthophyll*

Aliquots No.[1]	Saponifications	Washings with 92-percent methanol	Carotene found per gram of sample	Additional loss as compared to aliquots 1 and 2
	Number	*Number*	*Micrograms*	*Percent*
1 and 2	4	4	22.8	
3 and 4	8	4	21.9	3.9
5 and 6	4	8	22.7	.4

[1] 1 hay sample was ground and extracted, and aliquots of the extract were analyzed as indicated in the table.

After a ligroin solution of chlorophyll was saponified and then washed with methanol as decribed in method A, no visible color remained in the ligroin phase. Apparently this procedure completely removes the chlorophyll.

To sum up, the methods described above for the determination of carotene in feeds may fail to extract a small amount of the carotene (at most 3 or 4 percent); and an additional loss of possibly 2 or 3 percent ordinarily may occur in separating it from the chlorophyll and xanthophyll. The greater part of these losses of carotene may be avoided by re-extraction of the feeds and by extracting back in case of the saponifications and of the washings with 92-percent methanol. These losses have been disregarded in the data presented herein.

PIGMENTED IMPURITIES IN CAROTENE FRACTION FROM HAYS AND CORN SILAGE AFTER SAPONIFICATION AND SUBSEQUENT WASHING OF THE LIGROIN SOLUTION WITH METHANOL

The largest error in the estimation of the carotene in hays and corn silage by analytical methods in which the separation of the pigments is carried out solely by saponification, and subsequent washing of the ligroin solution of the carotene with 90- or 92-percent methanol, is apparently associated with the occurrence of colored impurities in the final carotene fraction that is used in the colorimetric or spectrophotometric measurements. The presence of such pigments is indicated by the biological activity of these carotene fractions, by their spectral absorption, and by their behavior when filtered through absorption columns. This evidence will be considered here.

The work of Hartman and associates (*12*) shows that when carotene extracts, prepared from pulverized alfalfa hay according to method A, are fed to rats that have been depleted of vitamen A, the resulting rate of growth is about 20 percent less than when the rats are fed doses of beta-carotene that were equivalent to the extracts in adsorption at wave length 436 mμ.

The data in tables 7 and 8 permit a comparison at certain wave lengths of the spectral absorption of various feeding materials with that of the alpha- and beta-carotene and leaf xanthophyll.

TABLE 7.—*Relative spectral absorption of xanthophyll, alpha-, and beta-carotene at various wave lengths of light expressed in percentage of the absorption at wave length 450 mμ*

Pigment	Relative spectral absorption at wave lengths of—						
	430 mμ	436 mμ	440 mμ	450 mμ	460 mμ	470 mμ	480 mμ
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Beta-carotene [1]	72.0	77.0	81.9	100	92.2	83.7	89.4
Alpha-carotene [2]	70.8	83.0	92.5	100	77.8	88.0	88.9
Leaf xanthophyll [3]	73.9	86.6	98.1	100	79.0	91.2	81.8
Second internation standard carotene [4]	72.3	.....	.....	100	93.1	82.7	[5] 87.3

[1] Data are averages calculated from charts by Miller (*31*), Smith (*40*), and McNicholas (*26*).
[2] Data are averages calculated from charts by Miller (*31*), and Smith (*40*).
[3] Data are averages calculated from charts by Miller (*31*), and McNicholas (*26*).
[4] Data were determined by the laboratory of the Bureau of Dairy Industry, by using spectrophotometer at color laboratory of Bureau of Chemistry and Soils.
[5] This low reading appears to be due to an error in the wave-length reading at 480 mμ, as results in accord with this assumption were obtained with color glasses standardized at the National Bureau of Standards, Washington, D. C.

TABLE 8.—*Relative spectral absorption of carotene extracts from various cattle feeds, expressed in percentage of the absorption at wave length 450 mμ*

Cattle feeds, from which carotene extracts were prepared [1]	Lots averaged	Spectral absorption at wave length of—		
		430 mμ	450 mμ	480 mμ
	Number	*Percent*	*Percent*	*Percent*
Second international standard carotene		72.3	100	87.3
Freshly cut green alfalfa		73.1	100	87.4
Artificially dried alfalfa meal	3	73.6	100	87.7
Alfalfa hay:				
U. S. No. 1	12	77.1	100	85.5
U. S. No. 3	4	79.8	100	82.2
Timothy hay:				
U. S. No. 1	6	78.5	100	84.7
U. S. No. 3	12	83.1	100	83.0
Corn plant chopped for ensiling		73.8	100	84.5
Corn silage	12	80.2	100	84.0

[1] Extracts prepared according to methods A and B described in the text. The hays were graded by representatives of the Bureau of Agricultural Economics.

If one allows for the variations that may result in such data from the use of different machines, different methods of reading, etc., it is obvious that the data for the carotene extracts from the freshly cut alfalfa and artificially dried alfalfa leaf meals resemble those for beta-carotene as nearly as can be expected; but that with the extracts from the hays and corn silage this is not true. With these extracts the relative absorption, as expressed in tables 7 and 8, is too high at wave length 430 mμ and too low at 480 mμ to correspond with either alpha- or beta-carotene and is not of a nature to be accounted for by assuming the presence of unremoved xanthophyll in the carotene. The discrepancy between the absorption of these extracts and that of the pure pigments is greater with extracts from the poorer quality hays.

The data in tables 9 and 10 show the results obtained when the carotene extracts from the hays and corn silage are filtered through adsorption columns.

TABLE 9.—*Effect of filtering the carotene extracts, prepared from hays and corn silage, according to methods A and B, through adsorption columns prepared from magnesium oxide*

Sample of feed analyzed	Wave length of light	Carotene extract not filtered through adsorption column		Carotene extract filtered through adsorption column				Pigmented impurity in unfiltered carotene fraction
				Carotene fraction from column		Impurity from column		
		Spectral absorption ratio [1]	Carotene, per gram of feed	Spectral absorption ratio [1]	Carotene per gram of feed	Spectral absorption ratio [1]	Equivalence in carotene, per gram	
	Millimicrons	*Percent*	*Micrograms*	*Percent*	*Micrograms*	*Percent*	*Micrograms*	*Percent*
Alfalfa hay, lot 85, U. S. No. 1	430	75.9		73.7		97.8		
	450	100.0	52.1	100.0	46.2	100.0	5.4	11
	480	85.2		87.6		68.2		
Alfalfa hay, lot 87, U. S. No. 3	430	79.7		[2] 74.4		95.5		
	450	100.0	3.2	100.0	2.4	100.0	.77	[2] 32
	480	85.1		[2] 86.2		74.2		
Timothy hay, lot 73, U. S. No. 1	430	75.3		73.6		93.0		
	450	100.0	32.2	100.0	28.7	100.0	3.6	
	480	84.6		87.2		72.7		11
Timothy hay, lot 79 U. S. No. 3	430	79.6		[3] 75.3		97.0		
	450	100.0	5.43	100.0	4.42	100.0	.94	[3] 30
	480	83.4		[3] 85.9		74.0		
Corn silage	430	76.7		72.8		86.7		
	450	100.0	[4] 42.4	100.0	33.0	100.0	9.6	23
	480	85.0		87.5		74.6		

[1] Expressed in percentage of the absorption at 450μ.
[2] The adsorption filtration apparently left about 7 percent of unremoved impurity still in the carotene fraction, making a total of about 32 percent of impurity in the original unfiltered sample.
[3] The adsorption filtration apparently left about 11 percent of unremoved impurity still in the carotene fraction, making a total of about 30 percent of impurity in the original unfiltered sample.
[4] Dry weight.

TABLE 10.—*Recovery of pigments in carotene extracts, prepared from hays and corn silage, when these extracts are filtered through magnesium oxide adsorption columns*

Sample of feed analyzed	Wave length of light	Absorbency readings under comparable conditions [1]			
		For carotene extracts filtered through adsorption columns			For unfiltered carotene extracts
		Carotene fraction	Pigmented impurity	Total	
	Millimicrons				
Alfalfa hay, lot 85, U. S. No. 1 [2]	430	0.813	0.127	0.940	0.940
	450	1.104	.129	1.233	1.239
	480	.969	.087	1.056	1.050
Alfalfa hay, lot 87, U. S. No. 3	430	.777	.317	1.094	1.105
	450	1.035	.332	1.367	1.381
	480	.905	.246	1.151	1.175
Timothy hay, lot 73, U. S. No. 1 [3]	430	.761	.122	.883	.872
	450	1.034	.131	1.165	1.159
	480	.903	.095	.998	.980
Timothy hay, lot 79, U. S. No. 3	430	.599	.164	.763	.778
	450	.796	.169	.965	.978
	480	.684	.125	.809	.816
Corn silage	430	.480	.178	.658	.658
	450	.657	.208	.865	.873
	480	.574	.162	.736	.723

[1] Samples comparable in size, final volume, and thickness of absorbing layer.
[2] 3 aliquots were filtered and the results averaged.
[3] 2 aliquots were filtered and the results averaged.

These adsorption columns were made from magnesium oxide (Merck). This material was activated by moistening, heating on a steam bath for 2 hours or more to bring it almost to dryness, and then heating for 1 hour in a muffle furnace at 200° C. It was ground through a 200-mesh sieve, and diluted with the same brand of siliceous earth used by Strain (*42*) to increase the rate of filtration.

The adsorption filtrations were carried out in an atmosphere of nitrogen (purified over copper) in an apparatus that was so arranged that all liquids could be boiled in a reduced atmosphere of nitrogen before coming into contact with the column. The column itself was only very mildly activated. This condition was selected after considerable experimentation, which indicated that with more active columns of this type there is a very considerable destruction of the pigments, including even the carotene. It has since been found desirable to cool these columns with ice water during filtration, especially during warm weather.

In these adsorption filtrations the pigments from the carotene extracts from the hays and corn silage separated readily into two fractions—one (the carotene) which was easily washed through the column with ligroin (boiling at 35° to 60° or 90° to 100° C.), and a second (the colored impurity) which adsorbed more strongly on the column and was eluted later with ligroin containing a small amount of ethanol. As shown in table 9, the former fraction when derived from corn silage or from hays of good quality, resembles beta-carotene very closely in spectral absorption; whereas the spectral absorption of the more strongly adsorbed fraction was such as to account for the difference, noted previously, between that of beta-carotene and the original unfiltered extract. With the poorer quality hays, it is evident that not

all of this colored impurity was removed from the carotene in the one filtration that was used.

Carotene extracts, prepared according to method B from freshly cut plant materials (alfalfa, Kentucky bluegrass, and corn plant) have likewise been filtered through magnesium oxide-adsorption columns. Although the spectral absorption of the pigments in the alfalfa and bluegrass extracts, both before and after the adsorption filtration, resembled that of beta-carotene closely and within the limit of the experimental error of the writers' methods of measurement, a small amount of pigmented impurity was removed by the filtration—about 7 or 8 percent in each case. The spectral absorption of the extracts from the corn plant before filtration indicated the presence of more pigmented impurity in them than in the extracts from freshly cut green alfalfa or bluegrass and more pigment was removed from these corn extracts as a result of the adsorption filtration. These results will be presented in a later paper in connection with a study of the relation of the pigments in corn silage to those in the plant material from which it was made.

From the data in table 10, it is evident that the separation of the carotene from the pigmented impurity by adsorption filtration was accomplished with these extracts from hays and corn silage with very little or no loss or alteration of the pigments present, although changes in absorption of the magnitude of those reported by Gillam and El Ridi (*6*) with this column would have been negligible in this connection and would not have been detected with the equipment that was used.

Although the results in table 9 indicate that 11 to 32 percent of the absorption of the unfiltered carotene extracts at wave length 450 mμ was due to colored impurities in them, these results do not furnish definite evidence as to the nature or source of this impurity. Its properties resemble those of complex carotene oxidation products, or possibly, especially with corn silage, a mixture of such substances with decomposition products similar to those reported, in the work referred to on page 638, to be formed by the action of acids on xanthophyll (*18*, *23*.)

Regardless of the nature of these impurities, the question naturally arises as to whether they occur in the hays and silages and whether a correction for them should be deducted from the writers' regular carotene figures, or whether this pigmented material has been formed in the process of analysis, possibly from carotene, and that therefore the writers' carotene figures are too low. This question is still not finally settled, but the evidence at hand favors the view that these colored impurities occur in the hays and silages, that they are not entirely absent even from freshly cut plant materials, and that they are not formed to a considerable extent in the process of analysis.

The pigmented impurity removed by adsorption filtration, as described previously, from the carotene extracts from the hays is readily altered when a more highly activated adsorbent than that made from magnesium oxide is used; but it would seem from the data in table 9 that under the above-mentioned conditions this impurity has very much the same spectral absorptive properties regardless of the kind or quality of the hay from which it is obtained. It is possible, therefore, to calculate roughly, using average absorp-

tion data, the proportion of the absorption at a given wave length (e. g., wave length 450 mμ) that is due, on an average, to the presence of this colored impurity in the original unfiltered carotene extracts from the different types of hay. The results of such calculations are shown in table 11. A similar calculation with the unfiltered carotene extracts prepared from corn silage, according to methods A or B, indicates that on an average the proportion of the absorption at wave length 450 mμ that is due to these colored impurities is 30 percent or more.

TABLE 11.—*Average percentage of the absorption at wave length 450 mμ that is due to pigmented impurities in carotene extracts prepared from hays and corn silage according to methods A or B*

Kind of feed and grade [1]	Lots of feed included in average	Average calculated impurity in carotene extracts
	Number	*Percent*
Alfalfa hay:		
U. S. No. 1	12	18
U. S. No. 3	4	38
Timothy hay:		
U. S. No. 1	6	24
U. S. No. 3	12	40
Corn silage	12	30

[1] Grades determined by representatives of the Bureau of Agricultural Economics.

One might, from calculations similar to the above, correct in a rough way by making the appropriate deduction from the results for the carotene content of hays and corn silage as determined by methods A and B. The data given in tables 15 to 21 are not thus corrected, for in their present form they seem to be comparable to similar data from other laboratories. They express roughly the relative amounts of carotene in these feeds and thus constitute a very useful and practical guide in feeding (p. 660).

COMPARISON OF DIFFERENT METHODS OF DETERMINING THE CAROTENE IN CATTLE FEEDS

A number of methods for determining the carotene content of cattle feeds have been used in different laboratories. Some of these methods have been compared in the Beltsville laboratory, and the results are presented here together with other work which indicates somewhat the extent to which results of this sort may be comparable.

METHODS OF ANALYZING FRESHLY CUT PLANT MATERIALS FOR CAROTENE

The writers have compared method B with other procedures for determining the carotene content of freshly cut plant materials, as follows:

Procedure No. 1.—Freshly cut alfalfa was immediately frozen with solid carbon dioxide in the field, then dried in a vacuum pan cooled with carbon dioxide, and ground and analyzed as in method A. This procedure gave 356 μg of carotene per gram of dry material, as compared with 362 μg for samples done by method B, in which alcohol that was not initially cooled was used. The difference is not significant.

Procedure No. 2.—Fresh green material was put in a vacuum chamber at 100° C. within 5 minutes of cutting, dried in a stream of air at this temperature and at 7-cm pressure, and then ground and treated as in method A. Three lots of alfalfa were analyzed in this way. This procedure gave an average carotene content of 323 μg per gram of dry matter, as compared with 343 μg for samples done by the same method B procedure as was used in procedure No. 1. When other samples were dried in a stream of air at 60° instead of 100° C., much lower results were obtained (72 percent lower in one case and 90 percent in another).

Procedure No. 3.—Peterson, Bohstedt, Bird, and Beeson reported (*34*) that they were able to extract more carotene from freshly cut alfalfa if they acidified the alcohol they used in extracting it. They furnished the writers a description of the method they used, which involved refluxing the freshly cut plant material with alcohol containing 0.5 ml of concentrated hydrochloric acid per 100 ml of alcohol. In table 12 are shown the quantities of carotene found in the alfalfa plant by the method used by Peterson, et al., by method B in which the material was initially frozen at −60° to −70° C., and by this latter procedure with 0.5 ml of concentrated hydrochloric acid added per 100 ml of the alcohol that was used in this freezing mixture. The results show that a very much lower figure was obtained for the carotene when acid was used in its extractions than with the usual procedure employed by the writers.

TABLE 12.—*Effect of the presence of mineral acids on the quantity of carotene found in freshly cut alfalfa*

Lot, or sample No., of alfalfa analyzed	Date of cutting	Carotene per kilogram found when sample was analyzed by—		
		Method B, using alcohol at −60° to −70° C.	Method B with 0.5 ml concentrated HCl added per 100 ml of ethanol, using alcohol at −60° to −70° C.	Method used by Peterson, Bohstedt, Bird, and Beeson (*34*)
		Milligrams	*Milligrams*	*Milligrams*
1	July 18, 1935	166	129	42
2	July 30, 1935	280	54	113

Very great difficulty was encountered in the ligroin-methanol separation with the acid-treated samples for which the results are given in table 12. The spectral absorption of these extracts was, therefore, determined at a number of wave lengths. This was also done with the pigments that were removed from the carotene fraction by the methanol in this separation after this carotene fraction had been washed sufficiently to remove the xanthophyll completely. These data are shown in table 13.

TABLE 13.—*Effect of the presence of mineral acids in the extraction of carotene from alfalfa; the spectral absorption of the carotene fraction and of the pigments extracted from it by 92-percent methanol after removal of the xanthophyll*

Alfalfa hay sample No.	Fraction or pigment tested	Procedure used in preparing fractions or pigments	Relative spectral absorption at wave lengths of—[1]					
			440 mμ	445 mμ	450 mμ	460 mμ	470 mμ	480 mμ
			Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1 and 2	Carotene fraction	(Method B, no HCl	82.4	98.1	100.0	92.8	84.9	87.1
		(Method B, plus HCl	107.3	100.0	100.0	85.2	75.3	76.0
2	Methanol extract [2]	do		102.0	100.0	83.3	79.6	77.8
1	Carotene fraction	Peterson, et al. (*34*)	101.5	99.0	100.0	82.2	75.1	71.1
2	do	do		100.0	100.0	98.4	83.4	85.5
1 and 2	Methanol extract [2]	do	89.3	102.3	100.0	79.8	79.3	73.4

[1] Expressed in percentage of the absorption at wave length 450 mμ.
[2] These methanol fractions were obtained by repeatedly washing the carotene fractions after the latter had been washed a sufficient number of times to completely remove the xanthophyll.

The results in table 13 leave no doubt that the material that was removed from the carotene in the washing of the acid-treated samples was not carotene; but indicate, instead, that considerable quantities of this same material still remained in the carotene fraction. On the other hand, the spectral absorption of the carotene fraction with the samples that were done by the regular procedure of the Beltsville laboratory, was, as pointed out previously, in very close accord with that for beta-carotene. Apparently, in this laboratory the use of acid leads to a destruction of carotene. This is in accord with the early observations on the effect of acids on carotene and xanthophyll (*44*); and the more recent work of Kuhn and coworkers (*18*, *23*) would lead one to expect in addition a similar—in fact a greater—alteration of the xanthophyll, and difficulty similar to that encountered, in removing these products from the carotene.

METHODS OF DETERMINING CAROTENE IN HAYS

Methods A and B have been compared with the following procedures for the determination of the carotene in hays:

Procedure No. 1.—Samples were done by method A, omitting the initial soaking in alcohol. Three lots of alfalfa hay analyzed in this way averaged 19.5 mg of carotene per kilogram (air dry), and by method A, 21 mg.

Procedure No. 2.—Samples were done by method A, omitting the initial soaking in alcohol, but using 1 percent of ethanol in the low-boiling ligroin used in extraction. Four lots of hay analyzed by this method averaged 18.7 mg of carotene per kilogram (air dry) and by method A, 18.5 mg.

Procedure No. 3.—The ground hay sample was extracted six times as in method A except that acetone was used in place of the ethanol and ligroin; and the carotene remaining in the hay residues was finally washed out with ether as described by Schertz (*38*). The acetone was washed out of this mixture by treatment with water (*38*), the ether removed in vacuo, and the pigments taken up in low-boiling ligroin, saponified, etc., as in method A. Three lots of hay analyzed in this way averaged 20.0 mg of carotene per kilogram (air dry), and by method A, 21 mg.

Procedure No. 4.—Hay that had been soaked in alcohol as in method A, was extracted with low-boiling ligroin in a Soxhlet extractor. Four lots of hay analyzed in this way averaged 11.4 mg of carotene per kilogram (air dry) and by method A, 12.3 mg.

Procedure No. 5.—Hay was analyzed by method B, except that the chlorophyll in the ligroin solution was saponified with an equal volume of 5-percent KOH-ethanol at 40° C. for 3 hours according to the procedure of Kuhn and Brockmann (*18*) for the saponification of xanthophyll esters. Six carotene extracts from alfalfa hays that were done in this way gave 3.45 μg of carotene per milliliter; and method B with four regular saponifications, gave 3.50 μg. With three samples of alfalfa hay that were saponified by the procedure of Kuhn and Brockman and by method A with four saponification treatments, the average figures were 3.94 μg and 3.72 μg per milliliter of extract, respectively. These results would indicate (1) that little or no xanthophyll ester occurs in the carotene extracts from alfalfa hays when analyzed by the methods used here; and (2) that the shaking and aeration in saponifying, as done in routine work here, is probably not harmful.

Procedure No. 6.—Ninety-percent was compared with 92-percent methanol in the ligroin-methanol separation. Samples that were washed eight times gave 31.1 mg and 30.6 mg per kilogram of hay, respectively. Other samples from this same hay (alfalfa) that were washed 15 times gave 30.3 mg and 27.7 mg, respectively. It is clear that the last figure is likely to represent most nearly the actual carotene content of this hay, as pigmented material was clearly visible in most of the additional 92-percent methanol washings, the amount of it far exceeding that under similar conditions with pure carotene and decreasing with successive washings as, of course, would not occur perceptibly with carotene itself.

Procedure No. 7.—Guilbert's method (*7*). In this procedure 95-percent ethanol (by volume) was used in preparing the alcoholic-potassium hydroxide solution. This alcohol was treated with silver nitrate and potassium hydroxide to remove aldehydes, although the error due to resins from this source appears to be negligible. The ether extract was not brought to "dryness," but was concentrated in vacuo to remove the ether. Besides washing finally with 90-percent methanol, as recommended by Guilbert, additional samples were done by using 92-percent methanol as is usual here. Controls were run with alcoholic potassium hydroxide alone to determine the spectral absorption of the resins from this source. The extracted hay residues were re-extracted and this extract analyzed in the same way as the original extracts. These controls and extracts from re-extraction were read at wave lengths 430, 450, and 480 mμ. Several lots of hay were analyzed; the results in table 14 for one lot of hay, are typical.

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TABLE 14.—*Comparison of results by Guilbert's method and by method B for the determination of carotene in hay*

[The extinction (E) is calculated to 1-cm layer for a 5-g sample and 50-ml volume]

Extracts, controls, and residues	Extinction coefficient and relative spectral absorption at wave lengths of—					
	430 mμ		450 mμ		480 mμ	
	E	Relative absorption [1]	E	Relative absorption [1]	E	Relative absorption [1]
Extracts washed 15 times:						
With 92-percent methanol:		*Percent*		*Percent*		*Percent*
Method B	0.524	79.8	0.657	100	0.536	81.6
Guilbert's method [2]	.540	79.1	.683	100	.550	80.5
With 90-percent methanol:						
Method B	.590	79.1	.746	100	.616	82.6
Guilbert's method [2]	.598	80.2	.746	100	.629	84.3
Resin in ligroin phase from KOH-ethanol control [3]	.065	130.0	.050	100	.024	48.0
Second extraction of hay residues: [4]						
Washed with 92-percent methanol	.026	81.2	.032	100	.027	84.4
Washed with 90-percent methanol	.048	85.7	.056	100	.042	75.0

[1] Expressed in percentage of the absorption at wave length 450 mμ.
[2] There is apparently no evidence of resinous material from the heating of the KOH and ethanol in these samples.
[3] Done by Guilbert's method, washing 15 times with the 90-percent methanol.
[4] Done by Guilbert's method in both cases.

It is obvious that this method and method B give very similar results with alfalfa hay when the extracts are similarly and adequately washed with methanol; the carotene is about equally completely extracted from the hay by these methods; and the discrepancies in the spectral-absorption data with these extracts as compared with those of beta-carotene, are practically the same by both methods. The data are from a hay that had lost about 50 percent of its carotene in storage since it was purchased, and the extracts with both methods obviously contain considerable amounts of pigments other than carotene. If these pigmented impurities had not been present in this hay, it is obvious from the data in table 14, showing the effect of using 90- and 92-percent methanol in the separation of carotene and xanthophyll, that this variation in the method of washing would have produced no difference in the results obtained.

CAROTENE CONTENT OF MARKET HAYS

ALFALFA HAYS

Table 15 shows the United States grade, color, and carotene content of 46 lots, or purchases, of alfalfa hay analyzed in the Beltsville laboratory. These hays were field-cured and baled. At the time of purchase they were inspected by representatives of the Division of Hay, Feed, and Seed of the Bureau of Agricultural Economics. The color, leafiness, time of cutting, and any other observations that were made regarding the condition of the hays, are noted in the table. The color and leafiness of some of the lots of hay was determined by inspection of the lot as a whole, but for other lots these factors were determined separately in the bales that were analyzed for carotene. The inspector examined some of these bales at Beltsville, but very

often they were sent to the laboratory of the Division of Hay, Feed, and Seed for more accurate examination.

TABLE 15.—*Carotene content of the various market grades of alfalfa hay*

PURCHASED AS U. S. NO. 1

Laboratory designation of each lot, or purchase, of hay	Natural green color	Leafiness	Stage of maturity at cutting	Carotene content per kilogram (air-dry basis)
	Percent	*Percent*		*Milligrams*
50	60–65	43	One-half in bloom	37
Test B [1]	85			121
Calf B [1]	75			37
57	72	56	One-fourth to one-half in bloom	31
58	73	51	Early bloom	35
59	65	50	do	31
64 [2]	60	40		43
H–O–1	70			41
H–O–1a	70			82
70				44
74 [3]	66	42	One-tenth in bloom	22
76	71	44	One-tenth to one-half in bloom	48
79	65	45	Full bloom	42
Mat. B., Oct. 17, 1936	85	45	do	63
Shop, medium, Nov. 5, 1936	67	40		35
Shop, low, Oct. 19, 1936 [4]	62	40		19
81	70	40–42	One-half in bloom	27
84	80	45	Bud	47
85	75	40	do	49
86	65–70	40–45	One-tenth to one-half in bloom	32
89	65–70	40	Full bloom	32
90				38
93				20
75: [5]				
Sublot A	75	45	One-fourth in bloom	57
Sublot B	65	43	do	8
Average	70	44	do	32
77: [6]				
Sublot A	70	45	One-half in bloom	48
Sublot B	60	30	Full bloom	17
Average	67	40		38
80: [7]				
Sublot A	70	48	One-fourth in bloom	54
Sublot B	67	42	One-half in bloom	72
Average	68	45		64
Average of 25 hays as purchased				42.7
Average of 27 hays that graded U. S. No. 1 in color only [8]				42.8

[1] Not graded officially; but classed as "very green."

[2] The bales in lot 64 contained some hay that was brown and that was graded U. S. No. 3. The No. 3 hay made up about 29 percent of the lot and contained 1.8 mg of carotene per kilogram. The remainder of the hay, an average of 70 percent per bale, was graded U. S. No. 1 and contained 60.6 mg of carotene per kilogram. The carotene content shown in the table is for a composite sample from 10 bales.

[3] Some of the bales in lot 74 were "set" or "packed" and looked as if they had been baled too soon. There were also yellow leaves that might have been the result of leafhopper infestation. The odor seemed normal.

[4] This sample had been stored from April to October in a rather dark barn loft.

[5] Lot 75 as purchased consisted of about equal portions of sublots A and B. The hay in sublot B had a sweated or ground odor, indicating that some decomposition or fermentation had occurred. There was no evidence of this condition in sublot A.

[6] Lot 77 as purchased consisted of two-thirds of sublot A and one-third of sublot B. There was no evidence of fermentation in sublot B.

[7] Lot 80 consisted of sublots A and B in the ratio of 15:19. The stalks in sublot A appeared to be finer and smaller than those in sublot B.

[8] Includes lot 73.

TABLE 15.—*Carotene content of the various market grades of alfalfa hay*—Continued

PURCHASED AS U. S. NO. 2

Laboratory designation of each lot, or purchase, of hay	Natural green color	Leafiness	Stage of maturity at cutting	Carotene content per kilogram (air-dry basis)
	Percent	*Percent*		*Milligrams*
60 [9]	45	45		19
H–O–2 [10]	55	56		14
H–O–2a	58	50		13
51	50–60	50		15
54	51	43		12
Mat. B. Nov. 12, 1936	45	40		20
73: [11]				
Sublot A	38	42		15
Sublot B	67	30		46
Sublot C	81	30		77
Average	63	34		46
Average of 7 hays as purchased				19.9
Average of 7 hays that graded U. S. No. 2 in color only.[12]				14.9

PURCHASED AS U. S. NO. 3

71.5	38	20		11
56	1	41		1
48	10–25	45	Bud to early bloom	11
51 [13]	15	25	In bloom	8
63 [13]	20	30	do	3
66	15–35	35–38		5
67	15–25	30–35		4
71 [13]	5–10	20–25	One-tenth in bloom	1
78	0–6	35–48	Bud	2
87	15	15	Early cut	3
88	20	15		1
91				4
92				4
Average of 13 hays as purchased				4.5
Average of 12 hays that graded U. S. No. 3 in color only.[14]				4.0

[9] Lot 60 as purchased was graded U. S. No. 1 (color 60 percent and leafiness 50 percent) but upon reinspection, the bales that were analyzed were graded as shown and they appeared to be representative of the lot. 1 bale that appeared "set" but not caked, indicating that fermentation had occurred in it, was graded 40 percent in color and contained 10 mg of carotene per kilogram. 2 other bales were caked, but in sampling them 1 bale had been drilled from an end that was much greener and less affected, if at all, by fermentation. These 2 bales contained 24 mg of carotene per kilogram.

[10] This was a single bale of hay. On Feb. 12, 1935, it was graded 63 percent in color and the carotene content was 14 mg per kilogram; on Apr. 5, 1935, it was graded 48 percent in color and the carotene content was 15 mg per kilogram. The datum in the table for color is the average of these determinations.

[11] Lot 73 was not uniform. The bales in sublot A looked as if the hay had heated in the bale as a result of bailing too soon after cutting. There were about equal amounts of each sublot.

[12] Omits lot 73 and includes lot 71.5.

[13] These hays were badly weathered.

[14] Omits lot 71.5.

The method of color measurement used in the Bureau of Agricultural Economics is described by Nickerson (*33*), who gives the Munsell hues associated with the various percentages of "natural green color," as expressed in hay inspection. According to the hay standards, described by the Bureau of Agricultural Economics (*43*), a sample of alfalfa hay may be sufficiently high in percentage of natural green color to be in grade 1, but may be placed in grade 2 or 3 because its leaf content is low (table 15, lot 73, sublots B and C; and lot 77, sublot B); or it may contain sufficient leaves to be in grade 1, but be placed in grade 2 or 3 because its percentage of natural green color is low (table 15, lots 60; H–O–2; H–O–2a; 73, sublot A; 51; 54; 56; and

48). Also a lot, or purchase, of hay is frequently not uniform, some bales being quite different from others (table 15, lots 75, 77, 80, and 73). The data in table 15 are according to the grading of the lot, or purchase, of hay as a whole; but, where the bales in the lot differed in this way, the lot, or purchase, of hay was separated into uniform sublots and each sublot was analyzed. The data for these sublots are also given in the table.

Attention should be called to lots 74; 75, sublot B; 60; and 73, sublot A. Fermentation or spoilage was evidenced in all of these hays by a caked or packed condition in the bale and by a tobacco, ground, or sweated odor.

In considering the data in table 15 one must bear in mind that besides the errors in the carotene determinations discussed above, there are relatively large and unavoidable errors in the determination of the leafiness and color of these hays. Despite these facts, an inspection of the data for the alfalfa hays in table 15, seems clearly to justify the following summary:

(1) There is a very decided difference between the average vitamin A potency of the No. 1, No. 2, and No. 3 grades of alfalfa hay as indicated by the carotene content, the average carotene content of the hays shown in table 15 being 42.7, 19.9, and 4.5 mg per kilogram, respectively, for these different grades. Since alfalfa hay is graded commercially mainly on the basis of its leafiness and color, the question arises as to whether these differences in carotene content are associated with either of these factors used in grading.

(2) The results in table 15 indicate that, although the carotene occurs largely in the leaves of the alfalfa plant (*36*) and is high in alfalfa leaf meals (table 16), the percentage of leafiness in field-cured, market hays plays a minor role in determining their carotene content. It is true that the average leafiness of the hays that are poorest in carotene is lower than the average leafiness of the others. Thus the average leafiness of the hays containing 1 to 11 mg of carotene per kilogram, as is the case with the grade 3 hays, is only about 30 percent, whereas the leafiness of the hays containing more than this amount of carotene is about 42 percent. Nevertheless, the average leafiness of the hays containing 12 to 20 mg of carotene per kilogram, as is the case with the grade 2 hays, is fully as great as that of the hays containing between 27 and 77 mg per kilogram; and there are hays that are low in their leaf content but high in carotene, and vice versa. Undoubtedly a large proportion of the carotene in these alfalfa hays occurred in the leaves, but the leaves, nevertheless, may or may not be rich in this pigment.

A few samples of machine-dried alfalfa leaf meals have been analyzed for carotene and the results are shown in table 16.

TABLE 16.—*The carotene content of machine-dried alfalfa leaf meals*

Sample No.	Greenness	Carotene content per kilogram (air-dry basis)	Sample No.	Greenness	Carotene content per kilogram (air-dry basis)	Sample No.	Greenness	Carotene content per kilogram (air-dry basis)
	Percent	*Milligrams*		*Percent*	*Milligrams*		*Percent*	*Milligrams*
1	97	155	4	74	76	7	80	212
2	64	122	5	85	244			
3	51	90	6	85	160	Average		151

The high carotene figures for these meals are in accord with results from other laboratories. The spectral absorption data in table 8 are of interest in this connection, being in good agreement with those shown for beta-carotene (second international standard, table 8).

(3) From data in table 15, it is obvious that any evidence of spoilage such as a caked or set condition in a bale of hay or a tobacco, sweated, or ground odor (table 15, lots 60; 73, sublot A; 74; 75, sublot B) may be taken as an indication of a decidedly low carotene content. This condition may be associated with a low percentage of green color (lots 60; and 73, sublot A); but apparently it also occurred in hays that were not graded particularly low in this respect (lots 74; and 75, sublot B).

This fermentation in hays is the result of baling them before they are sufficiently dry; but overexposure to sunlight in drying has also been found to be destructive of carotene and must be avoided if hays of high carotene content are to be obtained. The optimum conditions at which hays should be placed in storage have been studied by Shepherd and Woodward (*39*).

(4) The percentage of natural green color in alfalfa hays is by far the most important, general, and reliable index of the carotene content. This is obvious from the results shown in figure 1, as well as from those in table 15.

If one disregards all lack of uniformity and all evidence of fermentation in the lots of alfalfa hay shown in table 15 and grades these hays on the basis of color alone, the average carotene content of the hays placed in grade 1, 2, or 3 is 42.8, 14.9, and 4.0 mg per kilogram, respectively. The hays in grade 1 would on this basis contain 19 to 121 mg of carotene per kilogram of hay; those in grade 2, 11 to 20 mg; and those in grade 3, 1 to 11 mg.

On the other hand, if each uniform lot or sublot of hay shown in table 15 is graded according to its color, and those hays in which spoilage occurred are omitted, then the average carotene content of the hays placed in grade 1, 2, or 3 is 45.7, 14.2, and 4.1 mg per kilogram, respectively. The carotene content, on this basis, would vary from 17 to 121 mg per kilogram, 11 to 20 mg per kilogram, and 1 to 11 mg per kilogram, respectively, for grades 1, 2, and 3. These results are shown graphically in figure 1.

The two hays of lowest carotene content that on the latter basis would be in grade 1 are of interest. They are lot 77, sublot B and a single bale of hay designated as "Shop, low, Oct. 19, 1936." The former was graded barely 60 percent in green color and contained only 30 percent of leaves; the latter was from a lot of hay (lot 80, sublot A) that was purchased and first analyzed during April 1936. From April to October this hay had been stored in a rather dark barn loft. Under these conditions of storage the percentage loss of carotene is much greater than that of green color (*46*); and the bale that was analyzed in October is apparently no exception. It was 65 percent lower in carotene content, but only about 10 percent lower in green color than those bales that were analyzed in April. This result is the only one included in table 15 for a hay that was stored in this way after being purchased. It illustrates a general condition which tends materially to alter the relation between the color and the carotene content of alfalfa hays. Hay that has been stored under these

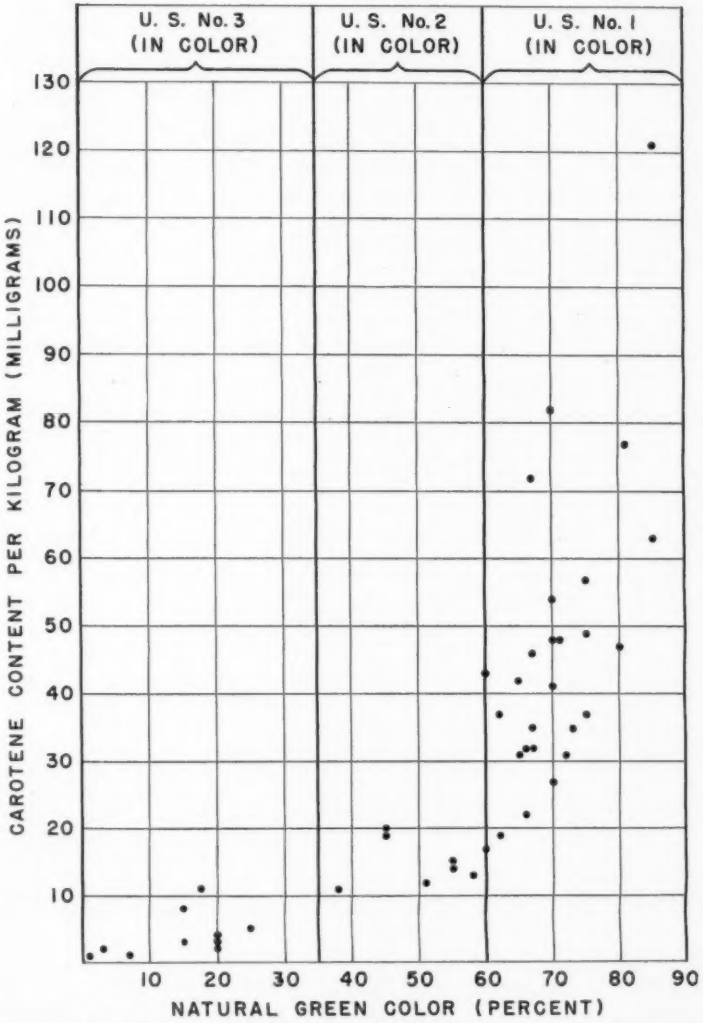

FIGURE 1.—Relation between the United States grade (in color only) and the carotene content of the alfalfa hays analyzed at the Beltsville laboratory. No hays in which spoilage had occurred are shown, and only uniform lots or sublots of hay are included.

conditions may have a much lower carotene content, as compared with its color, than it had soon after it was cut. Obviously, there is a very useful practical relation between the color and carotene content of alfalfa hays; but, of course, no definite quantitative relation between these factors can be expected.

It is interesting to note that all the 27 uniform lots or sublots of alfalfa hay with a "natural green" color of 60 percent or more (fig. 1) contain 20 mg or more of carotene per kilogram; and that a hay with this minimum concentration of carotene, when fed at about 50 percent of the ration, furnishes close to the minimum amount of vitamin A that a cow requires for normal reproduction (*35*) (p. 660). It is interesting to note further that before either of these facts was known, hay-grading authorities had selected this percentage of natural green color as the minimum for U. S. No. 1 alfalfa hay.

TIMOTHY HAYS

Table 17 gives the United States grade, color, and carotene content of 40 lots or purchases of timothy hay that have been analyzed in the Beltsville laboratory. Although the carotene content of the hays overlaps to some extent in the various grades, there is a distinct tendency for it to vary with the grade of the hay. The average carotene content of the timothy hays in grades 1, 2, and 3 is 20.6, 9.3, and 5.5 mg per kilogram, respectively. As the grading of timothy hays is based on the percentage of green color in them this means that, as with the alfalfa hays, the carotene content varies in general with the color. But the amount of this pigment varies from 8 to 36 mg per kilogram in the grade 1 hays; from 8 to 11 mg per kilogram in the grade 2 hays; and from 1 to 12 mg per kilogram in grade 3 hays. As would be expected, the timothy hays that were cut when ripe or overripe are uniformly low in their carotene content; whereas those hays that were cut in the prebloom stage generally contain more of this pigment.

TABLE 17.—*Carotene content of various market grades of timothy hay*

PURCHASED AS U. S. NO. 1

Laboratory designation of each lot	Natural green color	Stage of maturity at time of cutting	Carotene content per kilogram (air-dry basis)
	Percent		*Milligrams*
40	50		20
46	48–50	Prebloom	23
47	45	do	22
51	47	do	[1] 8
57	55	do	19
61	62	Bloom and early bloom	16
73	85	Late bloom	36
75	55–60	Before bloom	21
80	60–65		19
43:			
7 bales	50		21
1 bale	50		30
Average	50		22
Average of 10 lots			20.6

[1] Average of 8 agreeing determinations on 5 bales of hay. The bales were carefully graded and showed no evidence of spoilage. Meigs and Converse report (unpublished) that they found this hay inadequate in its carotene content to prevent the abnormal calving by a cow that received it as approximately 50 percent of her ration, from the sixty-first to the eleventh day before calving.

TABLE 17.—*Carotene content of various market grades of timothy hay*—Continued

PURCHASED AS U. S. NO. 2

Laboratory designation of each lot	Natural green color	Stage of maturity at time of cutting	Carotene content per kilogram (air-dry basis)
	Percent		*Milligrams*
39	35–40	Early dough	8
44	35–40	Quite immature	9
48	35	Early dough	11
Average			9.3

PURCHASED AS U. S. NO. 3

37	20–25	Ripe	1
42	15–20	Late dough	10
38	10		1
45	20	Ripe	4
53			2
56	15–20	Overripe	6
64	22	Ripe	8
65	8	do	7
66			5
60	15–20	Overripe	4
69	20–25	do	5
70	15–20	do	3
72	15–30	do	11
74	20	Ripe	12
76	10	Ripe (headed for seed)	4
77	10	Late dough, weathered	3
78	20	Late dough	5
79	10	do	5
81	20–25	do	2
82	15	Ripe	3
83	15	Late dough	4
84	20	Ripe	10
85	15	do	9
86	15	do	11
87	15–20	do	6
88	5	Ripe and weathered	3
89			5
Average			5.5

CLOVER HAYS

Only four samples of clover hay have been analyzed for carotene in the Beltsville laboratory. They were all graded at the time of purchase as either U. S. No. 1 Clover or U. S. No. 1 Clover Light Timothy Mixed. The carotene content of the four samples was 11, 14, 24, and 43 mg per kilogram of hay, respectively.

PRACTICAL SIGNIFICANCE OF CAROTENE DETERMINATIONS IN CATTLE FEEDING

Most of the hays for which the carotene content is given in this paper have been fed to cattle at the Beltsville station, largely in experiments in which they were practically the only source of vitamin A in the ration. Converse, Wiseman, and Meigs (*3*) have studied the effect of these hays upon the nutritive value of the milk of cows, while Meigs and Converse (*29*) have investigated the value of the hays as a source of the vitamin A required by dairy cows for various functions, particularly for growth and reproduction. Their results indicate that there is practically a definite relation between the

carotene content of hays (as determined by routine analytical methods) and the value of these hays as a source of vitamin A.

They found that the reproductive function in the cow is sensitive to a deficiency of vitamin A in the ration, and that the amount of vitamin A required for this function quite likely determines the minimum amount of vitamin A or its precursors that it is advisable to feed to a cow. On the other hand, it is clear from their work that there is a corresponding range of carotene content, as determined in the hays that they used, that meets these requirements. They report failure in reproduction with all of their cows, when U. S. No. 3 alfalfa or timothy hay constituted approximately 50 percent of the ration and was very nearly the only source of vitamin A in the ration for a period of at least 5 months before the cows calved. The calves were born dead, blind, or so weak that they died soon after birth. When U. S. No. 1 alfalfa hay was similarly fed, there was normal success in reproduction; and with hays that were intermediate in carotene content—possibly ranging from 10 to 20 mg or 15 to 20 mg of carotene per kilogram of hay—the results were variable.

In a subsequent report based on these experiments of Meigs and Converse, which are still in progress, Rogers (*35*) states:

> If the carotene content of the hay of the ration, where fed at about one-half of the ration, is as high as 20 parts per million calving is normal; if it is as low as 14 the results are uncertain and at levels as low as 5 parts per million there are no normal calvings.

Apparently, with cows fed as here described, those hays listed in tables 15 and 17 that contain 20 mg or more of carotene per kilogram, as determined by methods A and B, would supply close to the minimum amount of vitamin A required for normal reproduction; and those containing 14 mg or less would not do so. The carotene figure, therefore, as determined for a hay by the ordinary routine procedures described in this paper, is a decidedly useful index of practical value in cattle feeding.

As shown in the earlier part of this paper, this statement is true whether the carotene is determined according to methods A and B, described in this paper, or according to comparable methods developed in other laboratories. There is, therefore, available a considerable amount of comparable information on the suitability, from this standpoint, of various materials for cattle feeding and on the vitamin A requirements of cattle. However, just as numerous factors affect the efficient utilization of the energy or of the protein of the ration, the same may very well be found to be true of the utilization of vitamin A and its precursors (*13*), when these results are applied in practice.

In a recent paper, Guilbert, Miller, and Hughes (*9*) present the results of a study of the amount of carotene and vitamin A required by cattle to prevent night blindness. They report that a minimum of 25 to 30 μg of carotene per kilogram of body weight, or 6 to 8 μg of vitamin A, is necessary in the ration for this purpose; but they found that pregnant, nonlactating cows, maintained on these minimum levels throughout gestation, uniformly gave birth to weak calves that died soon thereafter. Increasing the carotene intake to three or four times this minimum during the last month of gestation consistently resulted in normal calves at birth. These workers also report that, with cows maintained for long periods during lactation on approxi-

mately the minimum level of carotene that would prevent night blindness, the milk was practically devoid of vitamin A and nursing calves developed symptoms of vitamin A deficiency and died.

This work clearly demonstrates that night blindness, as detected in cattle by the method that these workers used, is not a criterion by which to judge practically the adequacy of a ration as a source of vitamin A for dairy cattle, but that the minimum quantity of this factor that it is advisable to feed these animals certainly is more nearly the amount Meigs and Converse found to be necessary for normal calving (p. 660).

CAROTENE RESULTS WITH MISCELLANEOUS FEEDS

A number of miscellaneous feeding materials have been analyzed by means of the carotene methods described on pages 638–639 and the results are included in table 18. It must be borne in mind that the figures for the grains, although they include the carotene present, are not a measure of their vitamin A value.

TABLE 18.—*Carotene found in various feeding materials analyzed in the Beltsville laboratory*

Feeding material	Samples analyzed	Carotene per kilogram	
		Range	Average
	Number	*Milligrams*	*Milligrams*
Wheat straw (air dry)	1	----------	(1)
Corn stover (air dry)	2	2– 6	4
Kentucky bluegrass (dry weight)	4	424– 662	567
Alfalfa, freshly cut (dry weight)	9	271– 412	[2] 334
Garden carrots, yellow: [3]			
Green weight	11	36– 132	91
Dry weight	11	268–1,692	914
Corn grain, yellow	6	3– 9	[4] 5
Wheat bran	2	0.12– 0.23	[4] .18
Linseed meal	3	0.23– 0.29	[4] .26
Soybean meal	1	----------	[4] .23

[1] Less than 2 mg per kilogram.
[2] 1 sample of alfalfa, obtained from a plot that was infested by leafhopper, contained 166 mg per kilogram. The sample was selected as the best hay in the plot.
[3] Garden carrots purchased on market for feeding experiments.
[4] This figure includes the carotene along with other pigments, such as cryptoxanthine, which go more or less into the carotene fraction.

CAROTENE CONTENT OF CORN SILAGE

Since 1934, this laboratory has made about 30 determinations of the carotene content of the silage used in feeding the cattle in the herd employed in nutrition experiments at Beltsville. Each determination has consisted of the analysis of several aliquots, frequently from each of several large samplings. In some instances monthly composite samples have been made from samplings taken twice daily for the month, each daily sampling being kept in an ice box in a closed fruit jar until the monthly composite was made and analyzed. The results of these silage determinations are given in table 19.

TABLE 19.—*Carotene content of miscellaneous samples of corn silage*

Silage sample No.	Date of analysis	Carotene content per kilogram—		Silage sample No.	Date of analysis	Carotene content per kilogram—	
		On basis of silage as fed	On basis of dry weight			On basis of silage as fed	On basis of dry weight
		Milligrams	*Milligrams*			*Milligrams*	*Milligrams*
1	Mar. 8, 1934	12	48	20	Nov. 14, 1936	7	22
2	Mar. 14, 1934	12	48	21	Dec. 30, 1936	19	70
3	May 26, 1934	13	48	22	Feb. 10, 1937	18	60
4	July 9, 1934	3	13	23	Mar. 8, 1937	11	43
5	July 30, 1934	7	29	24	Mar. 15, 1937	9	28
6	Aug. 6, 1934	10	39	25	July 30, 1937	3	10
7	Sept. 4, 1934	16	60	26 [1]	March 1937	6	20
8	Sept. 17, 1934	11	39	27 [1]	April 1937	7	22
9	Nov. 17, 1934	1	4	28 [1]	May 1937	7	24
10	Jan. 5, 1935	37	146	29 [1]	June 1937	5	18
11	Mar. 4, 1935	40	156	30 [1]	July 1937	4	13
12	Mar. 9, 1935	28	111				
13	Mar. 16, 1935	29	103	Average		13.7	49.9
14	Mar. 21, 1935	36	122	Average, omitting samples 9 to 17, inclusive [2]		9.4	33.0
15	Mar. 25, 1935	10	35				
16	Sept. 5, 1935	25	98				
17	Sept. 20, 1935	8	29				
18	June 22, 1936	10	28				
19	June 25, 1936	7	20				

[1] Composite sample. 2 samples were taken from the silage as fed daily, kept in fruit jars in an ice box, and composited before analysis. The keeping of the samples in this way was tested out and was found not to lead to a destruction of the carotene. Later, however, evidence of spoilage was noted in some jars. On this account 4 of the 60 jars in April, 5 in May, and 1 in June, were discarded.

[2] Samples 9 to 17, inclusive, do not represent the results of average practice on the farm where this work was done.

The carotene content of the 30 samples of corn silage ranges from 1 to 40 mg per kilogram of the silage as fed, or from 4 to 156 mg per kilogram of dry weight The average carotene content of these 30 samples is 13.7 mg per kilogram of the silage as fed, or practically 50 mg per kilogram of dry weight.

All the samples of silage listed in table 19, except Nos. 9 to 17, inclusive, represent the results of average farm practice on the dairy farm at Beltsville. The carotene in these samples ranges from 3 to 19 mg per kilogram of silage as fed, or from 10 to 70 mg per kilogram of dry weight; and the average carotene content is 9.4 mg per kilogram of silage as fed, or 33 mg per kilogram of dry weight. These samples are probably not sufficiently comparable in other respects to permit any conclusions regarding the relation between their carotene content and the duration of the period of storage in the silo. This matter is now under investigation.

The data for the samples of silage that represent average farm practice at Beltsville agree with those from other laboratories, and probably represent the results obtained frequently in practice. Lipman (*24*) reports an average of 15 International units of carotene per gram of silage for six samples that were analyzed at the New Jersey Agricultural Experiment Station. Krauss (*16*) reported 2.5 Sherman units of vitamin A potency per gram for one sample of silage; and in a subsequent paper (*17*) reported using silage containing 52 mg of carotene per kilogram of dry material.

It is obvious from the results obtained with corn silage that frequently the silage might be suspected of failing to supply enough carotene in the ration to furnish the vitamin A required for normal

reproduction. Meigs and Converse have in progress in the Beltsville laboratory an experiment to test this point. No results of their experiment have yet been published, but they have been kind enough to permit the following statements: Up to the present time there have been five calvings by cows on a ration of grain and corn silage. The silage was fed ad libitum and the grain was fed in amounts to supply adequate energy and protein. Of these five calvings, three have been normal. In the other two cases the calves died at 2 and 13 days of age respectively.[7] Where corn silage is to be used thus in the ration of a cow as practically the sole source of vitamin A, or is to be depended upon in large measure to furnish this factor in the diet, it is evidently important to know the conditions under which it may be produced with adequate carotene content.

Preliminary work of this sort was started in the fall of 1934, with the silage samples 9 to 17, inclusive (table 19), to learn something of the conditions that determine the carotene content of corn silage. A record was available of the varieties of corn that had been planted in that year. When the crop was cut in the fall the maturity and greenness of the corn were noted, a record was kept of the way it was handled, the weather conditions, etc., and from time to time as the chopped corn was put into the silo a large amount of the material was mixed in the silo and duplicate samples were taken. Aliquots from one of these duplicates were analyzed immediately for carotene and moisture. The other duplicate, amounting to 15 to 20 kg, was placed in a very porous burlap bag, left in the silo, and analyzed when it was uncovered during subsequent feeding. Nine bags were thus left in the silos. The results of this experiment are shown further in table 20.

TABLE 20.—*Relation between the carotene content of corn silage and the maturity and carotene content of the corn from which it was made*

Silage sample No.[1]	Date of analysis of silage	Condition of corn plant at cutting		Carotene content per kilogram (dry-weight basis)	
		State of maturity	Greenness[2]	Corn plant[3]	Silage
			Percent	*Milligrams*	*Milligrams*
10	Jan. 5, 1935	Ears 100 percent in milk, undented	100	115	146
11	Mar. 4, 1935	do	90	92	156
12	Mar. 9, 1935	do	90	94	111
Average		do		100	138
13	Mar. 16, 1935	Ears 50 percent in milk, 50 percent in dough	90	70	103
14	Mar. 21, 1935	do	90	90	122
16	Sept. 5, 1935	Some milk, but average ear slightly dented	75	74	98
Average				78	107
15	Mar. 25, 1935	Ears dented	40–50	21	35
17	Sept. 20, 1935	do	40	25	29
Average		do		23	32
9	Nov. 17, 1934	Ears dented, plant exposed to light frost[4]	20	6	4

[1] Samples 10 and 11 were from the same variety of corn; samples 12, 15, and 17 were from the same variety; and samples 9 and 13 were from the same variety.

[2] The portion of the plant that was estimated to be green.

[3] Carotene content at time the material was chopped for ensiling. The time and condition of exposure between the time the corn was cut in the field and the time it was chopped for ensiling varied for the different lots of corn.

[4] There was a light frost for 1 night, 8 days before this corn was cut.

[7] In addition to these calvings on this grain-corn silage ration there was one abortion that occurred much earlier than those that have been associated here with a deficiency of vitamin A in the ration.

In considering the data in table 20 it will be recognized that a number of factors which may have affected the carotene content of the corn from which these silage samples were made varied—e. g., the variety of the corn, its maturity and greenness, the weather conditions preceding and at the time of cutting, the period elapsing between the time the corn was cut and the time it was put into the silo, the weather conditions during this time, etc. The carotene content as found in the samples of silage may have been affected, in addition, by variations in the time that the silage was stored, the exposure of the bags near the top of the silo before they were removed, and numerous other conditions. It is impossible from the information in this preliminary experiment to accurately evaluate or rigidly eliminate many of these factors; but, despite this situation, it is clear that certain correlations in the data in table 20 are much more consistent and striking than others. Of these the following may be noted:

(1) The carotene content of the corn from which the lots of silage were made was, as might have been expected, most strikingly and consistently affected by the maturity of the corn and the proportion of the plant that was green at the time of cutting. In the three greenest and least mature samples of corn the carotene content varied from 92 to 115 mg per kilogram of dry weight, and averaged 100 mg; in three samples of corn that were slightly more mature the carotene content varied from 70 to 90 mg per kilogram and averaged 78 mg; whereas in the two samples of corn that were fully dented (but not frosted) and only 40 to 50 percent green the carotene content was 21 and 25 mg per kilogram of dry weight, respectively. The effect of a light frost upon the carotene content of the corn plant is well shown in sample No. 9.

(2) The carotene content of the silage samples shown in table 20 was, without doubt, definitely affected by that of the corn from which they were made. Corn having an average carotene content of 100 mg per kilogram of dry weight produced a silage with a carotene content of 138 mg per kilogram; corn having an average carotene content of 78 mg produced a silage with a carotene content of 107 mg; and corn having an average carotene content of 23 mg produced a silage with a carotene content of 32 mg; while the sample of frosted corn with a carotene content of 6 mg produced a silage that contained only 4 mg. Samples 15 and 17 appear to represent the results of average practice in the making of silage on the farm at Beltsville.

(3) It may be noted that the carotene content of the silage samples in table 20, with the exception of the frosted sample, is uniformly higher than that of the corn from which they were made. The corn with an average carotene content of 100 mg per kilogram produced silage with a carotene content apparently 38 percent higher; and for the two lots of corn with average carotene content of 78 and 23 mg, respectively, the apparent increase in carotene in the silage was 37 and 39 percent, respectively. Probably, the good agreement in these figures is largely a matter of chance; but it does indicate that the apparent percentage increase in carotene in the silage does not vary materially with the amount of carotene in the original plant material.

There were probably a number of changes in the pigments in the corn plant from the time it was cut in the field to the time samples of the freshly chopped material were taken from the silo, and also from

this time until the silage itself was analyzed. The increase in the amount of pigmented material in the carotene extracts from these silages, as compared with the corn from which they were made, may possibly best be regarded as the net result of at least more than one change going on in these pigments in the silo, and insofar as the writers are aware, there is no conclusive evidence indicating the nature of the material making up this net increase of pigment in the carotene fraction from the silage. It is true that Krauss (*16*), reported 2.5 Sherman units of vitamin A potency per gram in silage made from corn that contained only 1.25 Sherman units; but no details of this work were given.

Apparent increases in carotene content, similar to those just noted with ordinary corn silage, have been reported repeatedly in silage made by the A. I. V. method,[8] where it would seem much less likely that carotene might be produced by bacteria or in the continued metabolism of the cells of the ensiled plant; and, although it has been demonstrated that A. I. V. silages are rich in carotene and are potent sources of vitamin A, no one seems to have determined the nature or biological properties of the additional pigment that occurs in the carotene extracts from them. It is possible that this additional pigment may be carotene; but since, according to Kuhn and Brockmann (*18*), pigments that behave like carotene in analysis may be formed by the action of weak acids on xanthophyll, one must regard such an assumption with some reservation until further evidence is at hand. Also, since a similar acid reaction prevails during the storage of corn silage, this possible origin of the increase in pigment in corn silage over and above that in the corn from which it was made must be borne in mind.

The data discussed on pages 643–648, indicating that the carotene extracts obtained in the routine analysis of corn silage contain pigments other than carotene and that these so-called pigmented impurities account on an average for about 30 percent of the absorption at wave length 450 mμ, are of interest in connection with the data in table 20, which apparently show that more carotene exists in the silage than was in the corn from which it was made. It is of interest, further, to note that the average amount of pigmented impurity in these extracts is about equivalent to this apparent increase in the carotene content of the silage. Thus, regardless of the nature of the changes in the pigments during storage in the silo, apparently the full amount of carotene originally in the corn is well maintained.

In considering the data in table 20 from a practical standpoint, it may be pointed out that to cut corn when the ears are practically all in the milk stage entails a considerable loss of yield in dry matter per acre. Cutting corn at this early stage is probably unnecessary, however, for it will be noted that corn that was cut in the early dough stage, or when the kernels were slightly dented, still yielded a silage that was rich in carotene. In silage from corn that was cut when the kernels were fully dented and only 40 to 50 percent of the plant

[8] In the A. I. V. method, named for its sponsor A. I. Virtanen of Finland, certain acids are added to increase the acidity of the ensiled material.

was green, the carotene content was considerably reduced; and the practice of cutting corn at this stage is likely to lead to the production of considerable silage that is inadequate in its carotene content to supply the amount of vitamin A required by dairy cows for normal reproduction, especially when it is practically the sole source of carotene in the ration. The minimum amount of carotene, as ordinarily determined in corn silage, that it is necessary to feed a cow has never been definitely determined. Certainly, it would be well to feed enough silage to provide at least 100 to 150 mg of carotene per day during the dry period, and more would certainly be desirable.

SUMMARY

The methods for the determination of carotene that are now in general use in the laboratory of the Division of Nutrition and Physiology at Beltsville, Md., are described in this paper. According to these methods the carotene is separated from the chlorophyll and xanthophyll by a modification of the well-known Willstätter and Stoll procedure.

Data are presented here to show the errors that occur in the use of this procedure. The largest error in the determination of the carotene in hays and corn silage by this procedure was found to be due to pigmented impurities other than xanthophyll or chlorophyll in the final carotene extracts. The presence of such pigmented impurities has been demonstrated by a study of the spectral absorption and adsorptive properties of these extracts. The error from this source in determining the carotene in the extracts varied from about 11 to 32 percent in the case of the hays and corn silage. It was least with fresh green plant materials and with leaf meals and hays of high carotene content. The source of these pigmented impurities is still uncertain. Some evidence tends to indicate that they are not in general produced in the process of analysis, but this possibility has not been satisfactorily excluded.

Despite this error in the determination of the carotene in feeds and the uncertainty of the source of these pigmented impurities, the carotene content as determined by the usual routine methods now in use in the Beltsville laboratory is shown to be a very useful index of the vitamin A value of feeds of plant origin that are used in cattle feeding.

Data are presented on the carotene content of various kinds and qualities of market hays, the relation between their physical properties and carotene content, the carotene content of corn silage, some of the conditions affecting the carotene content of this silage, and the results obtained in applying these routine carotene-determination methods to fresh green plant materials, alfalfa leaf meals, various grains, etc. These results are summarized in table 21.

TABLE 21.—*Summary of results on the carotene content of cattle feeds, given in this paper*

Feed	Carotene content per kilogram			
	Dry weight		Weight as fed	
	Range	Average	Range	Average
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*
Green growing material:				
Bluegrass	424–662	567		
Alfalfa [1]	271–412	332		
Artificially dried material: Alfalfa leaf meals			76–244	151
Silage: Corn	4–156	50	1– 40	14
Alfalfa hay:				
Grade U. S. No. 1 in color			19–121	43
Grade U. S. No. 2 in color			12– 20	15
Grade U. S. No. 3 in color			1– 11	4
Timothy hay:				
Grade U. S. No. 1 in color			[2] 8– 36	21
Grade U. S. No. 2 in color			8– 11	9
Grade U. S. No. 3 in color			1– 12	5
Carrots (yellow garden)	268–1, 692	914	36–132	91
Corn stover (dry)			2– 6	4
Clover hay (U. S. No. 1)			11– 43	23

[1] Samples not infested by leafhopper.
[2] 1 apparently exceptional sample contained 8 mg of carotene per kilogram.

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HOST-PARASITE RELATIONS IN RED CLOVER PLANTS RESISTANT AND SUSCEPTIBLE TO POWDERY MILDEW, ERYSIPHE POLYGONI[1]

By OLIVER F. SMITH[2]

Assistant agronomist, Division of Forage Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture

INTRODUCTION

Plants of red clover, *Trifolium pratense* L., show wide differences in susceptibility to the powdery mildew fungus, *Erysiphe polygoni* DC. Most plants are highly susceptible and the fungus develops profusely, producing an abundance of superficial mycelium and conidia. There are, however, occasional plants on which there is no development of the fungus beyond spore germination and penetration into the host. Between these two extremes are plants upon which the fungus develops with different degrees of severity but which do not lend themselves to a rigid classification because of the many gradations that exist between susceptibility and apparent immunity.

In papers on the powdery mildews little has been recorded on the interaction between host and parasite in resistant and susceptible plants within a species. Smith (*8*)[3] and Hirata (*1*) have described in some detail haustorial development of several powdery mildew fungi but only on congenial host plants. Salmon (*7*) and Neger (*2*) have described the behavior of powdery mildew fungi on noncongenial host plants, but in their work plants of one species were inoculated with mildew from plants of other species.

The object of these investigations was to obtain information on the nature of the interaction between the host *Trifolium pratense* and the parasite *Erysiphe polygoni* that would be of assistance in breeding red clover for mildew resistance. The different degrees of superficial development of the fungus on the host are obviously associated with a host-parasite relation that can be examined only by cytological methods. Therefore cytological studies have been made on the penetration of the fungus into the host and the fate of the infecting hyphae in the epidermal cells of plants showing different degrees of susceptibility or resistance. This work parallels some of that done on the rusts, but since the many papers dealing with host-parasite relations in that group of diseases have been comprehensively reviewed elsewhere (*5*, *6*) it seems needless to consider them here except as they have a bearing on these investigations.

MATERIALS AND METHODS

Plants selected for these studies were for convenience arbitrarily placed in three classes, namely, susceptible, moderately resistant,

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[3] Italic numbers in parentheses refer to Literature Cited, p. 682.

and highly resistant, according to the amount of mildew that developed on them as shown in figure 1.

On susceptible plants, the fungus developed profusely and produced an abundance of mycelium over the surface of the leaves. The plants classified as moderately resistant occupied positions between the extremes of susceptibility and resistance, and consequently the amount of fungus development on them differed considerably. On vigorously growing leaves of highly resistant plants there was no fungus development beyond spore germination and penetration into the underlying epidermal cell. On some plants in this class, mildew developed slightly on senescent leaves but a few individuals were observed on which no mildew developed at any stage of their life; the latter were considered immune. Highly resistant plants are quite rare and therefore studies on that class were restricted to a few individuals, whereas in the

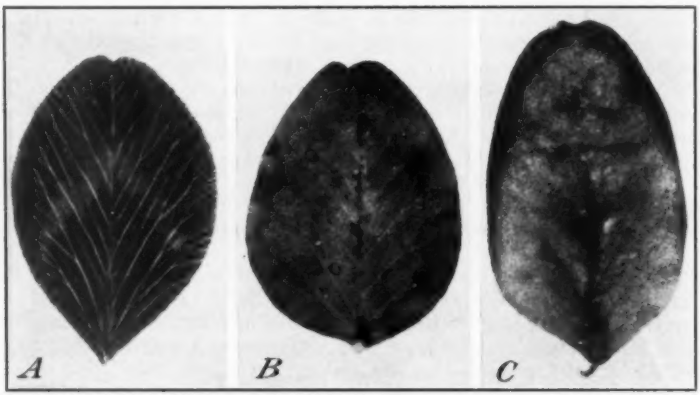

FIGURE 1.—Leaves from red clover plants artificially inoculated with *Erysiphe polygoni*: *A*, Leaf from a highly resistant plant, showing no fungus development; *B*, leaf from a moderately resistant plant, showing a moderate amount of fungus development and also a necrosis of leaf tissue following establishment of the parasite; *C*, leaf from a susceptible plant, showing abundant fungus development. × about 1½.

susceptible and moderately resistant classes samples of infected leaves were taken from a large number of plants. In all the work reported here, only young, vigorously growing leaves were used.

The plants were grown in the greenhouse and inoculated by brushing spores from mildewed leaves onto uninfected ones with a camel's-hair brush. Inoculations were made during March and April when abundant sunlight and a suitable greenhouse temperature, seldom exceeding 85° F., favored mildew development.

After inoculation, samples of leaf tissue were collected at 3-hour intervals for a period of 24 hours, then at 24-hour intervals for the next 3 days, and every fourth day thereafter for the following 2 weeks, and were fixed for 24 hours in Karpechenko's modification of Navashin's fixing fluid, consisting of equal parts of solution A (195 cc of water, 30 cc of glacial acetic acid, and 3 g of chromic acid) and solution B (195 cc of water, 30 cc of formalin (37 percent formaldehyde)) mixed just before using.

The process of dehydration and infiltration with paraffin was done according to the schedule given by Rawlins (*4, p. 21*), by the use of cedar oil following a series of alcohols of various concentrations up to 95 percent. Sections were cut 6μ and 8μ in thickness. Staining with safranine and fast green gave satisfactory differentiation between host and parasite. Sudan IV and ruthenium red were used for staining the cuticle and cell walls.

A modification of the cleared-leaf method described by Peace (*3*) was used for observing spore germination and early stages of infection on the leaves. Bits of leaf tissue were fixed in equal parts of alcohol and acetic acid until all the green pigments were removed; they were then stained in acid fuchsin in lactophenol, rinsed in alcohol-acetic acid (1:9), and cleared in chloral hydrate for examination.

Counts of spore germination on leaf tissue were made with the aid of an Ultrapak microscope.

Since physiologic forms of *Erysiphe polygoni* on red clover have been reported (*9*) questions may arise regarding the purity of the mildew used. Unfortunately the plants by which these physiologic forms were differentiated no longer exist. The plants used for these studies were consistent in the amount of mildew that developed on them and in host-parasite relations, and it is therefore assumed that physiologic forms did not confuse the results.

SPORE GERMINATION AND EARLY STAGES OF INFECTION

Observations have shown that there are no differences in percentage of spore germination or in the manner in which spores germinate, form appressoria, and effect penetration on the three classes of plants. Therefore the description of these processes applies to plants in any of the three classes.

On susceptible plants 600 conidia out of 691 observed had germinated in 20 hours after inoculation; on moderately resistant plants 472 had germinated out of 575 observed, and on highly resistant plants 618 had germinated out of 698 observed. The percentage germination on the three classes of plants was, therefore, 86, 82, and 88, respectively.

Gross observations on spore germination and on early stages of infection were readily obtained on cleared leaves stained with acid fuchsin. Under favorable conditions spores begin to germinate about 3 or 4 hours after being freed from conidiophores and coming to rest on the surface of a leaf. A germ tube grows out from one end of the spore and forms a well-defined appressorium (fig. 2, *A* and *E*), which becomes firmly attached to the leaf surface. Subsequently an infection hypha enters the underlying cell and an infection pore is apparent at the point of penetration (fig. 2, *B* and *F*). The appressoria are of various shapes and sizes and may form anywhere on the surface of an epidermal cell, but are most frequently located at the edge of a cell or over the walls which separate these cells. They sometimes form over stomata, but infection hyphae have never been observed to enter through these openings. A septum is formed between the appressorium and the spore (fig. 2, *C*). Under favorable conditions a spore germinates, forms an appressorium, and effects penetration in about 5 to 7 hours.

Detailed observations on penetration were made on microtome-sectioned leaf tissue. After an appressorium is attached firmly to the surface of the leaf a slender infection hypha begins to penetrate the cuticle and outer cell wall. During the very early stages of penetration there is an apparent swelling of the subcuticular materials for some distance around the point of penetration, which causes a separation of the cuticle from the cell wall. Figure 3, *A* and *F*, page 676 shows

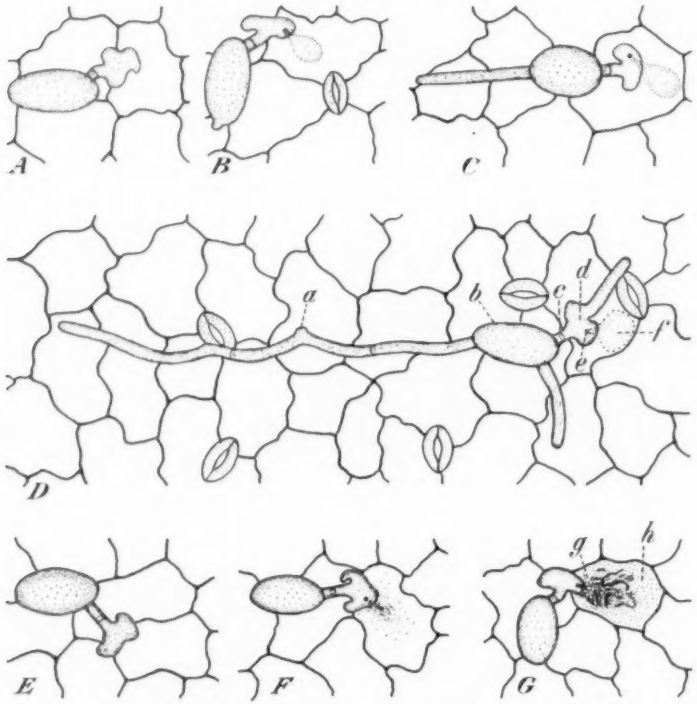

FIGURE 2.—Camera-lucida sketches of spore germination and early stages of infection by *Erysiphe polygoni* from cleared leaves of red clover: *A*, *B*, *C*, and *D*, On a susceptible plant; *E*, *F*, and *G*, on a resistant plant. *a*, Young appressorium from which penetration probably would have taken place into the underlying epidermal cell; *b*, spore; *c*, germ tube; *d*, appressorium; *e*, infection pore; *f*, haustorium and surrounding sheath; *g*, infection peg in (*h*) necrotic cell. × about 385.

infection hyphae that have pierced the cuticle and are in the swollen subcuticular area. There is no apparent alteration of the cuticle. Other stages of infection are shown in figure 3, *B* and *G*, where the infection hyphae have passed through the subcuticular region, entered the cell wall, and are somewhat enlarged at their distal ends.

Concurrent with these early stages of penetration, an ingrowth from the cell wall, which appears to be an addition of new materials

to its inner surface, develops at each point of penetration. In some cases the ingrowth extends over a third of the distance across the infected cell; in others it is barely perceptible. The amount produced is usually a little less than that shown in figure 4, *C* page 678. While an infection hypha is still within the cell wall and the ingrowth, a halo area (fig. 4, *B*) is often visible immediately around its tip, which indicates that these materials have been dissolved by the fungus as it penetrated into the cell. The plasma membrane passes up the sides and over the end of this structure and is later invaginated by the invading fungus.

INFECTION OF SUSCEPTIBLE PLANTS

An infection hypha, after making its way through the ingrowth of the cell wall, develops into a simple haustorium. This absorption organ consists of a slender neck, which passes through the wall of the epidermal cell and enlarges into a vesicular, distal portion, the ingrowth of the wall forming a collar which surrounds and accompanies the neck for some distance into the cell (fig. 3, *D* and *E*). The haustorium is uniseptate, uninucleate, and surrounded by a well-defined wall.

Around the haustorium is a conspicuous thick sheath containing a considerable amount of dark-staining, somewhat granular material, which is frequently gathered into irregular masses varying in extent and outline. This material often makes it difficult to see the septum in the haustorium. No evidence was obtained concerning the nature of this granular substance.

Surrounding the haustorium sheath is a conspicuous membrane which in early stages sometimes appears to be connected with the ingrowth of the cell wall, but in later stages becomes separated from the ingrowth and thus establishes itself as being independent of that structure (fig. 3, *E*). It is difficult to determine the exact nature of this sheath membrane. In early stages it is very thin and undoubtedly consists largely of plasma membrane, but in older stages it appears too thick to consist of plasma membrane alone, as suggested by Smith (*8*), unless the plasma membrane has become decidedly thickened along that portion surrounding the sheath. It appears more likely to have been produced by the plasma membrane of the host. This is in agreement with observations of a number of other investigators whose works have been reviewed by Rice (*5*, *6*).

The host cell nucleus moves toward the invading fungus. While stained slides were being examined for successive stages in the development of haustoria it was noted that the host nucleus was nearly always located near the invading fungus and had in all probability migrated to that position. In entire leaf tissue which was cleared and stained the host nucleus was never located away from the haustorium sheath, whether the haustorium was at the center of the cell or at one side of it. This is apparently the usual relationship between host nucleus and fungus haustorium, as Rice (*5*) lists 23 cases of habitual contact against 9 cases of occasional contact and 3 negative cases.

On susceptible plants there is no apparent antagonistic reaction between host and parasite. The fungus develops rapidly, and the contents of the invaded host cell appear to be as well organized as the contents of noninfected ones.

On a susceptible host, a spore germinates, forms an appressorium, penetrates into the host, and develops a haustorium in about 9 hours after inoculation (fig. 2, *B*). Another hypha then grows out from the other end of the spore (fig. 2, *C*) and develops into branching

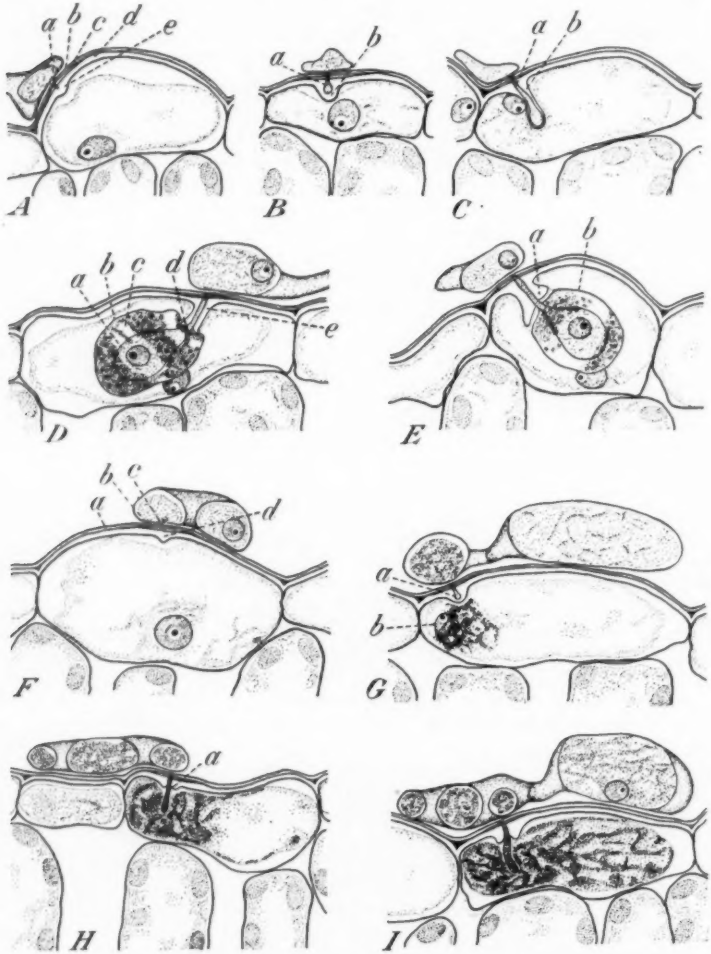

FIGURE 3.—FOR EXPLANATORY LEGEND SEE OPPOSITE PAGE.

mycelium which spreads over the surface of the leaf, forming appressoria and developing haustoria in the epidermal cells of the host. Additional mycelium may also develop from the spore and in some cases from the first-formed appressorium. Figure 2, *D*, shows an 18-hour-old infection where the spore has formed a haustorium in an epidermal cell, produced a small amount of mycelium, and formed

another appressorium at *a*, where penetration into the underlying cell with the formation of a haustorium is expected. The mycelium continues in this fashion to spread over the surface of the leaf, forming appressoria and haustoria and eventually conidiophores and conidia. Sporulation begins about 5 or 6 days after inoculation. Leaves of a congenial host plant will support a heavy growth of mildew for about 3 weeks, after which the leaves gradually become irregularly chlorotic and die.

INFECTION OF HIGHLY RESISTANT PLANTS

On a highly resistant host the parasite seldom develops further than the formation of an appressorium and the production of an infection hypha (fig. 2, *G*). The germ pore is formed on the lower side of the appressorium and an infection hypha enters the underlying epidermal cell as shown in figures 2 and 3. As the infection hypha enters the host cell there is a decidedly antagonistic reaction between host and parasite. The cytoplasm of the host stains heavily and appears to have undergone some degree of disorganization around the point of infection. In some instances this reaction is in evidence even before penetration into the cell is complete. Figure 3, *G*, shows an early stage of infection where the cell contents stained heavily before the fungus had actually entered the cell. Because of the small size of the young infection hypha it is impossible to determine whether there is a similar effect on the parasite at this stage of development but, if so, it is not severe enough to stop the progress of the fungus. As the infection hypha grows into the cell protoplast, apparently by an invagination of the plasma membrane, the antagonistic reaction becomes more intense (fig. 3, *H*) and within 48 hours after inoculation the entire contents of the cell and the infection hypha become disorganized masses of dark-staining granular materials (fig. 3, *I*) and the infected cell, as well as the spore and appressorium, collapses (fig. 4, *G* and *H*). An infection hypha seldom develops into more than a slender infection peg (fig. 4, *F*), although a few instances have been ob-

EXPLANATORY LEGEND FOR FIGURE 3

FIGURE 3.—Camera-lucida sketches of infection of epidermal cells of leaves of red clover by *Erysiphe polygoni: A, B, C, D,* and *E,* On susceptible plant; *F, G, H,* and *I,* on resistant plant. × about 930.

A.—Infection hypha (*a*) has pierced cuticle (*b*) and swollen subcuticular area (*c*) and entered cell wall (*d*). Note incipient ingrowth of cell wall at *e*.

B.—Infection hypha (*a*) has entered ingrowth of cell wall (*b*) and is slightly enlarged at its distal end. Nucleus of host cell is near point of penetration.

C.—Infection hypha (*a*) has passed nearly through ingrowth of cell wall (*b*).

D.—Fully developed haustorium (*a*) with sheath membrane (*b*), sheath (*c*), and septum (*d*). Ingrowth of cell wall (*e*) forms collar around neck of haustorium. Host penetration and development of haustorium are accomplished in about 9 hours after inoculation.

E.—Infection about 12 days old, showing haustorium sheath membrane (*b*) separated from ingrowth of cell wall (*a*). Note granular material in haustorium sheath in *D* and *E*.

F.—Infection hypha (*c*) has pierced cuticle (*a*) and is nearly through swollen subcuticular area (*b*). Note incipient ingrowth of cell wall at *d*.

G.—Infection hypha (*a*) is nearly through ingrowth of cell wall, and host cytoplasm has stained deeply near point of penetration. Host nucleus (*b*) is near point of penetration.

H.—Infection hypha (*a*), which stained heavily, has entered cell and is surrounded by heavily stained granular materials of host cell.

I.—Infection approximately 36 hours old. Infection hypha and cell contents are granular, have stained heavily, and are apparently dead.

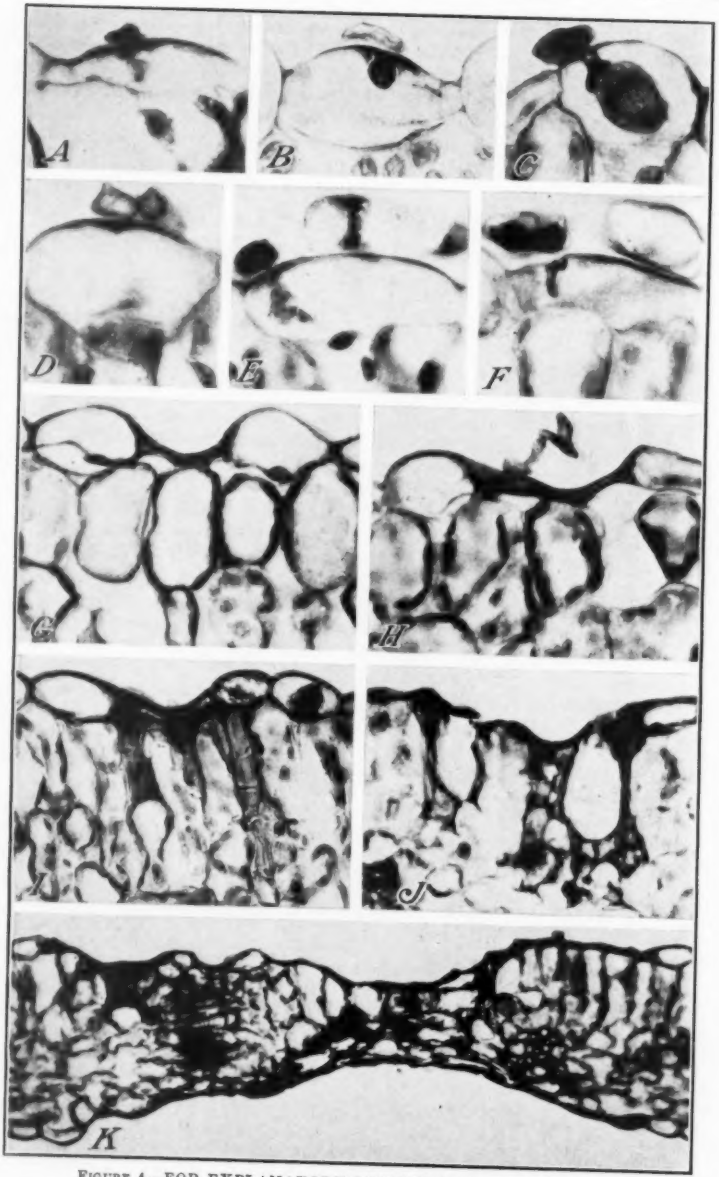

FIGURE 4.—FOR EXPLANATORY LEGEND SEE OPPOSITE PAGE.

served in which the infection hypha had become slightly enlarged at its distal end before the progress of the fungus was checked. Working with certain biological forms of *Erysiphe* and resistant hosts, Salmon (*7, p. 220*), found that incipient haustoria were sometimes formed but were—

> arrested and gradually disorganized under the influences at work in the cell of the "wrong" host-plant, or if the haustorium attains its full size it is hindered by these influences from carrying on its normal functions, and thus supplying to the fungus the food necessary for the production of mycelium, etc.

Injury to cells surrounding the infected one differs with individual plants. In some cases only the infected cell becomes necrotic, with no apparent adverse effects on the surrounding cells (fig. 4, *G* and *H*). In other cases cells adjacent to the infected one are killed and small brown necrotic areas are produced which are readily observed with the aid of an Ultrapak microscope but which cannot be seen macroscopically. A few plants have been observed in which sufficient cells became necrotic around each point of infection to produce macroscopically visible flecks on the leaves. It seems that in plants in which only the infected cell is killed, the host-cell contents become coagulated before the effects of the antagonistic interaction between host and parasite are transmitted to the surrounding cells. In plants that show death of a few cells in addition to the infected one, the effects of the host-parasite interaction are probably transmitted to those cells and cause their death. Whatever factor or factors may cause this antagonistic reaction, they are severe with highly resistant plants, less severe with plants possessing an intermediate degree of resistance, and apparently lacking with highly susceptible plants. Cytological investigations have not revealed their identity.

In resistant plants there is evidence also that the host cell nucleus moves toward the invading hypha. It was difficult to identify the

EXPLANATORY LEGEND FOR FIGURE 4

FIGURE 4.—Photomicrographs of different stages of infection on leaves of red clover plants by *Erysiphe polygoni*.

A, Early stage of infection on susceptible plant, showing infection hypha in ingrowth of cell wall. × about 880.

B, Early stage of infection on susceptible host, showing halo region around end of infection hypha in ingrowth of cell wall. × about 880.

C, Fully developed haustorium, with its sheath and sheath membrane, in epidermal cell of susceptible plant; × about 880.

D, Early stage of infection on resistant plant, showing very early development of ingrowth of cell wall and swelling of subcuticular materials which has caused a separation of cuticle from cell wall around point of penetration. Infection hypha has pierced cuticle and is in swollen subcuticular area. × about 880.

E, Young infection on resistant plant, showing dark-stained cytoplasm immediately below infection hypha, which is still in ingrowth of cell wall. × about 880.

F, Infection approximately 36 hours old on resistant plant. Infection hypha has entered host cell but has only developed into a slender peg. Both infection hypha and cell contents are granular and have stained heavily. × about 880.

G, Infection 7 days old on highly resistant plant. Infected cell has collapsed; no apparent injury to surrounding cells; × about 590.

H, Infection 20 days old on highly resistant plant. Infected cell has collapsed; no apparent injury to surrounding cells. × about 590.

I, *J*, and *K*, Infections 8, 12, and 18 days old, respectively, on moderately resistant plant that showed extensive necrosis of leaf tissue following infection. *I*, Infected cell has collapsed; underlying palisade cells are necrotic. Infected cell at right had not collapsed when this material was fixed. × about 400. *J*, Several epidermal and palisade cells have collapsed. × about 400. *K*, Necrosis and collapse of cells has occurred through leaf. × about 250.

host nucleus after the early stages of infection because of the disorganization of the host protoplasm, but when identifiable it was near the invading fungus.

INFECTION OF MODERATELY RESISTANT PLANTS

Between the extremes of susceptibility and resistance are plants upon which the fungus develops with different degrees of severity but which cannot be rigidly classified because of the many gradations that exist. Furthermore, experience has shown that when light is abundant the development of mildew on these plants increases, but with decreased light there is a smaller amount of mildew. However, the plants considered here as showing an intermediate or moderate degree of resistance did so under the seemingly optimum conditions for mildew development in which the highly resistant and highly susceptible plants were studied. On plants approaching a high degree of resistance, growth of the fungus is barely perceptible to the unaided eye and very few conidia are produced. On plants of greater susceptibility, growth of the fungus increases and there is also an increase in the number of conidia produced. Sectioned leaves of these plants showed that haustoria are produced in the epidermal cells, but on the more resistant plants they are not as large as those in epidermal cells of highly susceptible plants. With the exception of one plant, which will be considered later, there was little indication of adverse effects from host-parasite reaction on infected host cells except on plants approaching a high degree of resistance. In these cases the cytoplasm stained a little darker than in susceptible plants and appeared to be granular and somewhat disorganized.

One plant was observed that showed a distinct type of host reaction (fig. 1, *B*), but because mildew developed on it only in moderate amounts it is considered here with the class of moderately resistant plants. When this plant was inoculated, the fungus became established in a manner similar to that described for susceptible plants, and maintained a seemingly congenial relationship with the host plant for a short period. A considerable amount of mycelium and many spores were produced, sporulation beginning about 6 days after inoculation. After about 8 days, small brown necrotic spots developed on the leaves. At their earliest stages of development these spots involved only the infected epidermal cell and the underlying palisade cells (fig. 4, *I*). These spots increased in size quite rapidly, and about 12 days after infection several epidermal and palisade cells collapsed (fig. 4, *J*). Eighteen days after infection these necrotic areas extended entirely through the leaf (fig. 4, *K*), and because of increase in size they coalesced, and the death of the leaf soon followed. There can be little doubt that infection of epidermal cells initiated the development of these spots, as leaves of this plant kept free of inoculum showed no signs of necrosis.

Sectioned leaves of this plant showed that the collapse of cells was preceded by the deposition of a yellowish substance chiefly along the walls, first in the infected epidermal cell, then in the surrounding cells. By the time the infected epidermal cell had collapsed, the deposition of this substance and cell necrosis had extended into the underlying palisade cells (fig. 4, *I*). Necrosis and collapse of palisade and spongy parenchyma cells continued to spread throughout the

tissues in the vicinity of the infected cell (fig. 4, *J*) and eventually the necrotic area extended throughout the leaf (fig. 4, *K*). It appears that the antagonistic reaction between host and parasite was not severe enough to cause immediate death of either and that during the prolonged interaction an opportunity was afforded for the effects of this interaction to be transmitted to surrounding cells, causing reactions within them which eventually brought about their death.

SUMMARY

Results are reported of cytological investigations on infection and development of *Erysiphe polygoni* on susceptible, moderately resistant, and highly resistant red clover plants.

Early stages of infection were the same on the three classes of plants. Spores germinated, formed appressoria, and penetrated directly into epidermal cells of the host leaf. Concurrent with the early stages of penetration, an ingrowth of the wall developed at each point of penetration.

On susceptible plants an infection hypha, after entering a cell, developed into a simple haustorium. This absorption organ consisted of a slender neck that passed through the wall of the epidermal cell and enlarged into a vesicular distal portion, the ingrowth of the cell wall forming a collar that surrounded and accompanied the neck for some distance into the cell. The haustorium was surrounded by a thick sheath containing a considerable amount of dark-staining, somewhat granular material. This sheath was in turn surrounded by a conspicuous membrane.

On highly resistant hosts, an infection hypha entered an underlying epidermal cell, but its progress was soon stopped because of an antagonistic reaction between host and parasite. The cytoplasm of the host stained heavily and appeared to undergo some degree of disorganization around the point of infection, in some cases even before penetration into the cell was complete. As an infection hypha grew into the cell protoplast the antagonistic reaction became more intense, and finally the entire contents of the cell, as well as the infection hypha, became disorganized masses of dark-staining material and the infected cell ultimately collapsed. In some plants only the infected cell became necrotic, with no apparent adverse effects on the surrounding cells. With other plants, however, cells adjacent to the infected one became discolored and small brown necrotic areas were produced.

On moderately resistant plants, the fungus established itself in much the same manner as on highly susceptible ones and was able to maintain what appeared to be a fairly congenial relationship with the host plants. Growth on the surface of the plant varied with individuals, being roughly in proportion to the degree of susceptibility possessed by the plant. One plant was observed that showed an extensive necrosis of leaf tissue following infection. The fungus established itself and maintained a seemingly congenial relationship with the host for a period of about 8 days, after which necrotic spots appeared on the leaves. These increased in size, coalesced, and eventually killed the leaf.

The host nucleus moved toward the invading fungus in both resistant and susceptible plants.

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DIFFERENTIATION OF WOOD-DECAYING FUNGI BY THEIR REACTIONS ON GALLIC OR TANNIC ACID MEDIUM [1]

By Ross W. Davidson, *associate mycologist*, W. A. Campbell, *assistant pathologist*, and Dorothy J. Blaisdell, *junior pathologist, Division of Forest Pathology*,[2] *Bureau of Plant Industry, United States Department of Agriculture*

INTRODUCTION

In a study of only a few wood-decaying fungi Bavendamm [3] found that those species causing white decay formed a dark diffusion zone or "corona" under the fungus mats when grown on media containing small amounts of gallic or tannic acid, whereas those causing brown checked decay gave no such reaction. The brown diffusion zone was considered to be the result of oxidation of the acids, and hereafter this reaction will be referred to as "oxidase" reaction. Bavendamm also pointed out the effect of various concentrations of the acids on growth of the several species and suggested the use of such reactions for identification purposes should they be found consistent for a greater number of species.

Campbell [4] recorded the oxidase reactions of species of *Fomes* on tannic acid medium and found them to be consistent with Bavendamm's prediction.

It is the purpose of the present paper to give results of repeated tests of a greater number of species, representing several families and numerous genera, mostly of the Hymenomycetes. The above-mentioned tests have been used to assist in developing a method of identifying pure cultures of the wood-decaying fungi. Many such fungi have been isolated from decay in living trees on which no sporophores occurred. Such a test as the oxidase reaction, which separates the species into two distinct groups, is, when combined with numerous macroscopic and microscopic characters, very useful. The fact that the media used for the oxidase test also have a toxic effect on some species and not on others makes them of even greater value for identification purposes.

Growth rates, as well as oxidase reactions, of the fungi have been recorded on media of only one concentration, namely 0.5 percent of gallic or tannic acid added to plain malt agar as recommended by Bavendamm, although it is realized that other concentrations might give additional information of value.

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[2] In cooperation with the Civilian Conservation Corps. The authors express their appreciation to Dr. L. O. Overholts, of the Pennsylvania State College and Agricultural Experiment Station, for his interest and cooperation.
[3] Bavendamm, W. Über das vorkommen und den nachweis von oxydasen bei holzzerstörenden pilzen. Zuschr. Pflanzenkrank. u. Pflanzenschutz 38: [257]–276, illus. 1928.
[4] Campbell, W. A. The cultural characteristics of the species of fomes. Bull. Torrey Bot. Club 65: 31–69, illus. 1938.

SOURCE OF CULTURES

The cultures of the different fungi, the majority of which are wood-decaying, used to determine the oxidase reaction of the various species, were those previously identified and maintained in a collection by the Division of Forest Pathology.[5] Most of the isolates were from tissue of sporophores that had been carefully identified, many of them by L. O. Overholts, or from incipient decay with which identifiable sporophores were associated. Since isolates from these two sources react alike in culture, no indication is given in the list as to what proportion of the cultures used for each species were from sporophores. A few may not be definite wood decayers, but since they were associated with dead or decaying wood, they are included to make the study as complete as possible.

METHODS

The oxidase test is made by growing the fungus to be tested on malt agar to which gallic or tannic acid has been added. Gallic acid medium was prepared by the addition of 0.5 percent of gallic acid (Mallinckrodt, U. S. P. crystallized) to malt agar (1.5 percent of Difco malt with 2 percent of agar). Twenty grams of powdered agar and 15 g of Difco malt were dissolved in 850 cc of water in a 2-liter flask. One hundred and fifty cubic centimeters of water was placed in a separate flask. The dissolved malt agar and the flask of water were autoclaved for 20 minutes at a pressure of 15 pounds. Five grams of gallic acid was dissolved in the flask of sterilized water after removal from the autoclave. Heating gallic or tannic acid with agar causes hydrolysis of the agar; therefore the two cannot be autoclaved together. When the malt agar had cooled so that it could be handled without burning the hands, the gallic acid solution was added and thoroughly mixed. The resulting gallic acid medium was then quickly poured into 9-cm Petri dishes, about 35 cc to each dish. The dishes were spread in a single layer to insure rapid cooling. Gallic acid does not change the color of malt agar. Tannic acid medium was prepared in a similar manner, using 0.5-percent tannic acid (Mallinckrodt, U. S. P., powdered). This medium has a milk-white appearance.

In order to determine the effect of old and young mycelium on gallic and tannic acid media, two series of tests were made in which 1-year-old cultures and 2-month-old cultures, respectively, were used. Cultures of these different ages were used to see whether the older cultures had accumulated oxidases, which might produce stronger reactions. Results indicated that age of cultures beyond 2 months made little difference in reaction or rate of growth, as long as the cultures were still vigorous. Reactions are reported as averages, while in the case of growth the usual range in diameter of mat is given.

Gallic and tannic acids appear toxic to many fungi, and conclusive results seemed to depend upon the growth of the fungus on the media. For this reason large pieces of inoculum, 4 to 6 mm square, with as

[5] A number of the cultures in the reference collection were contributed by L. O. Overholts, Irene Mounce, Clyde Christensen, and Ray R. Hirt.

much fungus material as possible, were placed, mycelium down, on the agar and pressed gently in order to make close contact. When working with slow-growing fungi, from one to three inoculations of the same species were made in a single dish. Several commercial brands of gallic and tannic acids were tested to determine whether different sources of these acids would give comparable results. In general, the oxidase reactions were consistent but the growth rates varied considerably. Because of evident differences in commercial gallic and tannic acids, when comparing growth and to a lesser extent oxidase reactions of different fungi, it is important to use acids from the same commercial sources.

All the fungi, with the exception of a few recent acquisitions were tested for the oxidase reaction at least three times over a period of 2 years. The reactions and, to a lesser extent, growth (table 1) have appeared relatively consistent.

Observations upon the intensity of reaction and growth of mycelium were made at the end of 7 days for cultures kept in diffused light at room temperature (about 25° C.). In doubtful cases the cultures were kept for an additional 7 days. Most fungi that reacted with the acid media gave conclusive results at the end of 7 days. However, a number produced strong diffusion zones at the end of 1 or 2 days.

RESULTS

REACTION AND GROWTH DATA

The appearance and relation of the brown diffusion zone to the fungus mat, as well as the time required for its development, varied widely with different species. The following system was used to record the reactions of the different species to gallic and tannic acid media:

−, Negative, no brown discoloration of the agar under or about the mat.[6]

+, Diffusion zone light to dark brown, formed under inoculum at center of mat and visible only from under side of dish. In case no growth takes place, a faint brown discoloration under the inoculum.

++, Diffusion zone light to dark brown, formed under most of mat but not extending to margin. Visible from under side only.

+++, Diffusion zone light to dark brown, extending a short distance beyond the margin of the mat and visible from the upper side.

++++, Diffusion zone dark brown, opaque, extending considerably beyond margin of fungus mat.

+++++, Diffusion zone very intense, dark brown, opaque, forming a wide corona about mat. Usually such intense reactions occur with species giving no growth on the medium, and are most common on gallic acid medium.

Usually the brown diffusion zone which formed under the fungus mat would conform to one of the foregoing types. Occasionally a species gave a reaction that did not definitely fall into any of these groups; in such cases special mention is made.

The species of fungi showed interesting growth differences on malt agar containing 0.5 percent of gallic or tannic acid. These differences were caused by the toxicity of the acids. The following system

[6] Certain negative reactors cause decolorization of the medium under the mat. This decolorized zone, which forms most distinctly on tannic acid medium, should not be confused with the brown diffusion zone of typical reactors.

was used to record growth data: 0, no growth; tr., trace, growth confined to inoculum; 10, 15, etc., colony diameters in millimeters. Since growth rates were taken at room temperatures and are variable, they are expressed as a range rather than as an average.

Analysis of growth and reaction data at 7 days showed that the different fungi tested on gallic and tannic acid media could be classified into 10 fairly distinct groups. The behavior group for each individual fungus is given in the last column in table 1. Since this classification is based on growth in relation to type of reaction, no attempt was made to classify those species giving both positive and negative reactions on the same medium. A description of the groups and the number of fungi in each are given on pages 689–693.

TABLE 1.—*Reaction and growth of wood-decaying fungi on gallic and tannic acid media, at the end of 7 days*

Fungus	Type of decay	Isolations	On gallic acid medium		On tannic acid medium		Growth and reaction group No.[1]
			Reaction	Growth	Reaction	Growth	
		Number		*Mm*		*Mm*	
Thelephoraceae:							
Coniophora cerebella Pers.	Brown	5	−	30–50	−	20–35	1
C. suffocata (Peck) Massee	do	2	−	45–60	−	15–20	2
Corticium confluens Fr.	White	2	+++	0	++++	Tr.	4
C. coeruleum (Schrad.) Fr.	do	1	[2]− to +	30	+	Tr.	9
C. galactinum (Fr.) Burt	do	2	+++++	Tr.	+++	20–40	6
C. hydnans (Schw.) Burt		1	++++	0	+++	10	5
C. invrestiens (Schw.) Bres.	White	1	+++++	Tr.	+++	20	5
C. lividum Pers.	do	3	++++	25–45	+++	20–30	7
Cutidia salicina (Fr.) Burt		1	+++	0	+++	Tr.	4
Hymenochaete agglutinans Ellis		2	++++	Tr.–30	+++	30–50	8
H. corrugata (Fr.) Lév.	White	1	+++++	15	+++	45	8
H. curtisii (Berk.) Morgan	do	1	+++++	20	++++	20	7
H. rubiginosa Dick. ex Lév.	White pocket.	6	++++	Tr.–15	+++	Tr.–10	7
H. tabacina Sow. ex Lév.	White	5	+++++	20–35	++	20–50	7
Peniophora allescheri Bres.	do	1	++++	0	+++	Tr.	4
P. cinerea (Pers.) Cooke	do	2	+++++	Tr.	+++	35	6
P. coccineo-fulva (Schw.) Burt.		1	++++	35	++	Tr.	9
P. gigantea (Fr.) Massee		1	[2]+	10	−	0	9
P. incarnata (Pers.) Karst.		2	+++++	Tr.–20	++++	25–40	8
P. nuda (Fr.) Bres.	White	1	+++++	15	+++	35	8
P. pubera (Fr.) Sacc.	do	3	+++++	15–40	+++	20–60	7
Stereum albobadium (Schw.) Fr.	do	1	+++++	Tr.	+++	50	6
S. fasciatum Schw.	do	5	++++	15–40	+++	20–50	7
S. frustulosum (Pers.) Fr.	White pocket.	13	−	25–45	−	20–35	1
S. fuscum Schrad. ex Quél.	White	3	++++	Tr.–10	−	0	9
S. gausapatum Fr.	do	12	++++	10–25	+++	25–35	7
S. murrayi (Berk. and Curt.) Burt	do	6	++++	0	+++	Tr.–10	5
S. pini Fr.	do	2	+++++	Tr.	+++	20–40	6
S. purpureum Pers.	do	4	++++	0–tr.	+++	25–40	6
S. rameale Schw.	do	10	++++	20–50	+++	35–60	7
S. roseo-carneum (Schw.) Fr.	do	1	+++++	Tr.	+++	25	5
S. rugosiusculum Berk. and Curt.	do	1	+++	0	++	40	6
S. sericeum Schw.	do	1	++++	Tr.	+++	25	5
S. subpileatum Berk. and Curt.	White pocket.	10	[3]− to ++	10–15	−	10–20	----------
S. spadiceum Fr.	White	3	++++	Tr.–20	+++	25–40	6
S. sulcatum Burt.	White pocket.	1	+++++	0	+++++	10	5
S. umbrinum Berk. and Curt.	White	1	[3]− to +	35	+++	25	7
Hydnaceae:							
Echinodontium tinctorum Ell. and Ev.	do	5	++++	0	+++	Tr.	4
Hydnum caput-ursi Fr.	do	2	++++	0	++	0	4
H. coralloides Scop.	do	2	++++	0	++	0	4
H. erinaceus Bull.	do	20	++++	0	[4]− to +++	0–tr.	4
H. ochraceum Pers.	do	1	+++	0	++	0	4
H. pulcherrimum Berk. and Curt.		2	------	65–80	------	0	3
H. septentrionale Fr.	White	4	++	0	[4]++++	Tr.	4

See footnotes at end of table.

TABLE 1.—*Reaction and growth of wood-decaying fungi on gallic and tannic acid media, at the end of 7 days*—Continued

Fungus	Type of decay	Isolations	On gallic acid medium		On tannic acid medium		Growth and reaction group No.
			Reaction	Growth	Reaction	Growth	
		Number		*Mm*		*Mm*	
Hydnaceae—Continued.							
Irpex cinnamomeus Fr.	White.	3	++++	Tr.-10	+++	25-35	6
I. mollis Berk. and Curt.	do.	6	+++	0	+++	20-30	6
Phlebia strigosozonata Schw.	do.	3	+++++	0	++++	20-30	6
Radulum orbiculare Fr.	do.	1	++++	0	+++	Tr.	4
Polyporaceae:							
Daedalea ambigua Berk.	do.	3	+++++	0	++++	15-20	5
D. confragosa (Bolt.) Fr.	do.	5	++++	0	+++++	10-30	5
D. juniperina Murr.	Brown.	5	-------	20	-------	Tr.	3
D. quercina (L.) Fr.	do.	13	−	20-30	−	20-30	1
D. unicolor (Bull.) Fr.	White.	3	+++	0	+++	20-35	6
Favolus canadensis Klotz	do.	2	++++	0	++++	10	5
Fistulina hepatica (Huds.) Fr.	Dark firm.	15	−	15-20	−	10-25	1
Fomes annosus (Fr.) Cooke	White.	5	++++	0-tr.	+++	Tr.-10	5
F. applanatus (Pers.) Gill.	do.	22	+++	0-10	++++	10-20	5
F. calkinsii (Murr.) Sacc. and Sacc. D.	do.	4	++++	0-tr.	++++	10	5
F. conchatus (Pers.) Gill.	do.	9	+++	0-tr.	+++	Tr.	4
F. connatus (Weinm.) Gill.	do.	2	++	0	[4]+	0	4
F. densus Lloyd.	do.	4	+++	Tr.-10	+++	0-tr.	7
F. everhartii (Ell. and Gall.) Schrenk.	do.	26	++++	0	++++	10-15	5
F. fomentarius (L.) Gill.	do.	12	+++	0	++++	10-20	5
F. fraxineus (Bull.) Cooke.	do.	2	+++	0	++++	15-20	5
F. fraxinophilus (Peck) Sacc.	do.	9	++++	Tr.-10	[4]+++	Tr.-15	7
F. fulvus (Scop.) Gill.	do.	1	++++	Tr.	+++	25	5
F. geotropus Cooke.	do.	2	[2]− to +++	35-40	[2]− to +	10-15	9
F. igniarius (L.) Gill.	do.	6	++	0	+++	Tr.	4
F. igniarius var. *laevigatus* (Fr.) Overh.	do.	4	+++	0-15	++++	Tr.-20	5
F. igniarius var. *populinus* (Neu.) Campb.	do.	6	+++	Tr.-10	+++	Tr.	4
F. lobatus (Schw.) Cooke.	do.	6	+++	0-10	+++	10-30	5
F. marmoratus Berk. and Curt.	do.	2	++++	0	++++	10-15	5
F. meliae (Underw.) Murr.	Brown.	2	−	20-45	−	15-30	1
F. officinalis (Vill.) Faull.	do.	2	[5]−to+	10	−	0	-------
F. ohiensis (Berk.) Murr.	White.	1	+++++	0	++++	0	4
F. pini (Thore) Lloyd.	White pocket.	13	++++	Tr.-15	+++	Tr.-15	7
F. pinicola (Sw.) Cooke.	Brown.	26	−	15-30	−	10-20	1
F. rimosus Berk.	White.	10	++	0-10	++	0-tr.	4
F. robustus Karst.	do.	6	++++	Tr.	++++	10-25	5
F. robustus var. *tsugina* (Murr.) Overh.	do.	3	+++++	0-tr.	++++	Tr.	4
F. roseus (Alb. and Schw.) Cooke.	Brown.	11	[5]−to++	15-25	[5]−to+	Tr.-15	-------
F. scutellatus (Schw.) Cooke.	White.	4	++++	0	+++	0-15	5
F. subroseus (Weir) Overh.	Brown.	3	[5]−to+	25-30	−	10-25	-------
F. tenuis Karst.	White pocket.	3	+++++	0	++++	Tr.-10	5
Sterile *Fomes* on birch.	White.	13	+++	0	+++	Tr.-15	5
Lenzites betulina (L.) Fr.	do.	3	++++	Tr.	+++	30-45	6
L. saepiaria (Wulf.) Fr.	Brown.	6	−	10-15	−	Tr.	3
L. striata (Sw.) Fr.	do.	1	−	25	−	10	2
L. trabea (Pers.) Fr.	do.	4	−	20-30	−	0-tr.	3
Merulius confluens Schw.	White.	1	[2]+	20	−	0	9
M. tremellosus (Schrad.) Fr.	do.	7	+++++	0-40	++++	20-40	7
Polyporus abietinus (Dicks.) Fr.	White pocket.	1	++++	0	++++	Tr.	4
P. adustus (Willd.) Fr.	White.	6	[3]−to++	20-40	+	15-25	7
P. albellus Peck.	do.	6	+++++	0	++++	Tr.-10	5
P. alboluteus Ell. and Ev.	White[6].	1	+++	55	+++	60	7
P. amygdalinus Berk. and Rav.	Brown.	2	−	40	−	40	1
P. anceps Peck.	White.	10	+++++	0-tr.	[4]++++	Tr.-25	5
P. arcularius (Batsch.) Fr.	do.	1	++++	Tr.	+++	35	6
P. balsameus Peck.	Brown.	11	[5]−to+	15-25	−	0-tr.	-------
P. berkeleyi Fr.	White.	9	++++	0	++++	0-10	4
P. betulinus (Bull.) Fr.	Brown.	4	−	35	−	15-20	2
P. borealis Fr.	White.	3	++++	0	+++	0-tr.	4
P. brumalis (Pers.) Fr.	do.	4	++++	0	++++	20-30	6
P. cinnabarinus (Jacq.) Fr.	do.	8	+++++	0-tr.	++++	20-30	6
P. circinatus Fr.	do.	5	++++	0-tr.	+	0-tr.	4
P. compactus Overh.	do.	11	++++	15-30	+++	20-40	7

See footnotes at end of table.

TABLE 1.—*Reaction and growth of wood-decaying fungi on gallic and tannic acid media, at the end of 7 days*—Continued

Fungus	Type of decay	Isolations	On gallic acid medium		On tannic acid medium		Growth and reaction group No.
			Reaction	Growth	Reaction	Growth	
Polyporaceae—Continued.		*Number*		*Mm*		*Mm*	
P. conchifer (Schw.) Fr.	White	1	++++	0	++++	15	5
P. croceus (Pers.) Fr.	do	17	++++	10–35	++	20–40	7
P. curtisii Berk.	do	3	++++	10–20	+++	15–30	7
P. cuticularis (Bull.) Fr.	do	14	+++	0	++++	Tr.–15	5
P. delectans Peck	do	4	+++	0	+++	Tr.–10	5
P. dichrous Fr.	do	4	–	10	–	0	3
P. distortus (Schw.) Fr.	do	4	++++	10–15	+++	30–40	8
P. dryadeus (Pers.) Fr.	do	2	+++	0	+++	0	4
P. dryophilus Berk.	do	17	++++	10–25	+++	10–25	7
P. fibrillosus Karst.	Brown	1	–	15	–	15	1
P. fissilis Berk. and Curt.	White	12	++++	0	+++	Tr.–10	5
P. fragilis Fr.	Brown	4	–	10	–	0	3
P. frondosus (Dicks.) Fr.	White	10	+++	15–35	+++	10–35	7
P. fumidiceps (Atk.) Sacc. and Trott.	do	1	++++	Tr.	++++	20	5
P. fumosus (Pers.) Fr.	do	2	[2]–to+	15–30	[2]–to+	Tr.–15	7
P. galactinus Berk.	do	7	+++++	0–tr.	++++	Tr.–15	5
P. giganteus (Pers.) Fr.	do	4	+++	0	++++	10	5
P. gilvus (Schw.) Fr.	do	11	+++++	Tr.–15	+++	Tr.–30	5
P. glomeratus Peck	do	10	+++	0	+++	0	4
P. graveolens (Schw.) Fr.	do	8	[4]++	0	[4]+++	Tr.–20	5
P. guttulatus Peck	Brown	2	–	15–25	–	0	3
P. helveolus Rostk.		1	–	40	–	20	2
P. hirsutus (Wulf.) Fr.	White	7	+++++	0	+++	20–40	6
P. hispidus (Bull.) Fr.	do	15	++++	0–10	++++	10–20	5
P. immitus Peck	Brown	3	–	20	–	0	3
P. lucidus (Curt.) Fr.	White	14	++++	0–30	+++	15–40	7
P. ludovicianus (Pat.) Sacc. and Trott.	White pocket	[6]	+++	Tr.–10	+++	Tr.	9
P. mutabilis Berk. and Curt.	White	2	+++	0	+	0	4
P. obtusus Berk.	do	11	++++	0	++++	Tr.–15	5
P. oregonensis (Murr.) Kauff.	do	2	++++	0	++++	10	5
P. osseus Kalchbr.		1	–	55	–	0	3
P. palustris Berk. and Curt.	Brown	2	–	15–30	–	15–20	1
P. pargamenus Fr.	White	4	++++	0	++++	Tr.–15	5
P. picipes Fr.	do	1	++	0	+	0	4
P. pubescens (Schum.) Fr.	do	4	+++++	0	+++	30–40	6
P. radiatus (Sow.) Fr.	do	9	+++	Tr.–10	+++	Tr.–15	7
P. resinosus (Schrad.) Fr.	do	9	++++	Tr.–40	+++	10–35	7
P. robiniophilus (Murr.) Lloyd.	do	5	+++	0	+++	Tr.–20	5
P. sericeo-mollis Rom.	Brown	7	–	20–35	–	Tr.–10	3
P. schweinitzii Fr.	do	9	–	15–35	–	0	3
P. spraguei B. and C.	do	27	–	30–45	–	20–40	1
P. spumeus (Sow.) Horne	White	3	+++	0–tr.	++++	Tr.–20	5
P. squamosus (Huds.) Fr.	do	3	+++	0	+	0	4
P. subertypus (Murr.) Overh. in comb.	do	1	++++	0	+++	35	6
P. sulphureus (Bull.) Fr.	Brown	23	–	25–45	–	20–45	1
P. supinus (Sw.) Fr.	White	7	+++	0	++++	15–30	5
P. tenuis (Sacc.) Overh.	do	2	++++	0	+++	40–50	6
P. tephroleucus Fr.	Brown	2	[7]–	15	–	0	3
P. taugae (Murr.) Overh.	White	5	+++	0–tr.	+++	Tr.–25	5
P. tuberaster (Jacq.) Fr.		2	+++	0	+++	0–tr.	4
P. tulipiferus (Schw.) Overh.	White	9	[3]–to++	40–60	[3]–to+	Tr.–30	9
P. umbellatus (Pers.) Fr.		1	+++	0	+	0	4
P. velutinus Fr.	White	1	++++	0	+++	40	6
P. versicolor (L.) Fr.	do	6	+++++	0	+++	30–50	6
P. volvatus Peck	do	3	+++	0	++++	Tr.–10	5
P. vulpinus Fr.	do	1	+++	Tr.	+	0	4
P. zonalis Berk.	White pocket	5	++	0	[4]+++	Tr.–25	5
Poria andersonii (E. and E.) Neuman.	White	11	++++	0	++++	Tr.–10	5
P. borbonica Pat.	do	1	++++	10	+++	60	8
P. cocos (Schw.) Wolf	Brown	12	[5]–to+	50–80	–	30–80	
P. incrassata (Berk. and Curt.) Burt.	do	4	–	20–40	–	0–tr.	3
P. medulla panis (Pers.) Cooke.	White	2	+++++	Tr.–15	+++	30	8
P. mutans Peck	do	1	++++	20	+++	20	7
P. mutans var. *tenuis* Peck	do	1	++++	20	+++	30	7
P. nigra Berk.	Brown	3	–	25–35	–	25–35	1
P. nigrescens Bres.	White	3	++++	0	++++	Tr.–10	5

See footnotes at end of table.

TABLE 1.—*Reaction and growth of wood-decaying fungi on gallic and tannic acid media, at the end of 7 days*—Continued

Fungus	Type of decay	Isolations	On gallic acid medium		On tannic acid medium		Growth and reaction group No.
			Reaction	Growth	Reaction	Growth	
		Number		*Mm*		*Mm*	
Polyporaceae—Continued.							
P. prunicola (Murr.) Sacc. and Trott.	White	5	++++	Tr.–15	+++	Tr.–20	7
P. pulchella (Schw.) Cooke	do	5	+++++	Tr.–15	+++	15–50	8
P. punctata Fr.	do	6	+++++	0-tr.	++++	10–20	5
P. selecta Karst. ex Rom.	Brown	1	–	45	–	Tr.	3
P. subacida (Peck) Sacc.	White	14	++++	0–10	+++	10–30	8
P. undata (Pers.) Bres.	White pocket	3	++	0	+++	10–20	5
P. vaillantii Fr.	Brown	1	–	25	–	0	3
P. vaporaria Fr.	Brown [6]	2	–	30	–	0–10	2
P. versipora Pers.	White	1	++++	0	++++	15	5
P. weirii (Murr.) Sacc. and Trott.	do	2	+++	20–30	+++	20–30	7
Trametes americana Overh.	Brown	2	–	10–15	–	Tr.	3
T. cubensis (Mont.) Sacc.		1	–	70	–	0	3
T. heteromorpha (Fr.) Lloyd	Brown	1	[5]–to+	20	–	0	
T. hispida Bagl.	White	1	++++	0	++++	25	5
T. hydnoides (Sw.) Fr.	do	1	[3]–to++	25	+	10	9
T. malicola B. and C.	Brown	2	–	15	–	Tr.–10	1
T. mollis (Som.) Fr.	White	2	+++	0	++++	Tr.	4
T. rigida Berk. and Mont.	do	1	+++	0	++++	20	5
T. serialis Fr.	Brown	5	[7]–	15–40	–	10–30	1
T. suaveolens Fr.	White	3	++++	0	+++	10–30	5
Agaricaceae:							
Armillaria mellea (Vahl) Quél.	do	12	++++	0-tr.	++++	Tr.–10	5
Clitocybe illudens (Schw.) Sacc.	do	1	++++	10	++++	15	7
C. tabescens (Scop.) Bres.	do	6	++++	0–10	++++	Tr.–10	7
Collybia velutipes (Curt.) Quél.	do	4	+	0	–	0	4
Hypholoma sublateritium Schaeff.	do	6	++++	0-tr.	++++	Tr.–20	5
Lentinus lepideus Fr.	Brown	8	[5]–to+++	Tr.–15	[5]–to+	0-tr.	
L. tigrinus (Bull.) Fr.	White	4	++++	Tr.–25	+++	35–45	8
Panus laevis Berk. and Curt.	do	4	+++	0	++	0-tr.	4
P. stipticus (Bull.) Fr.	do	4	++++	0-tr.	++++	Tr.–20	5
Pholiota adiposa (Pers.) Fr.	do	8	+++	0	+++	Tr.–20	5
Pleuratus corticatus Fr.	do	4	+++	0	+++	0-tr.	4
P. ostreatus (Jacq.) Quél.	do	11	+++	0	[4]+++	Tr.–10	5
P. serotinus	do	2	+++	0–20	+++	Tr.–30	7
P. ulmarius (Bull.) Quél.	do	6	–to+	0	–	0	4
Schizophyllum commune Fr.	do	4	–	20–40	[3]++	25–40	10
Ascomycetes:							
Ustulina vulgaris Tul.	do	7	+++++	Tr.–20	+++	20–25	8
Xylaria sp.	do	2	+++++	Tr.	+++	30–40	6
Fungi Imperfecti:							
Strumella coryneoides Sacc. and Wint.	do	6	+++++	20–25	+++	30–50	8

[1] Growth and reaction groups are explained in detail in the text.

[2] These fungi may require from 7 to 14 days to form brown diffusion zones, but the reactions are always positive.

[3] Reaction uncertain; most isolations formed a brown diffusion zone under mat in 7 days. In 14 days zone more intense but certain isolations are consistently negative.

[4] Reaction positive if growth occurs; if no growth, usually positive but occasionally negative.

[5] Reaction usually negative. However, a faint brown diffusion zone may form under center of mat. The same isolation may be negative at one trial but slightly positive at another.

[6] SHOPE, PAUL FRANKLIN. THE POLYPORACEAE OF COLORADO. Ann. Mo. Bot. Gard. 18: 287–456, illus. 1931.

[7] Reaction negative. A widely diffused light-brown indefinite zone may appear around but not under mat. Agar under mat perfectly transparent.

NEGATIVE OR NONREACTING FUNGI

Group 1.—Mat diameter for a given species about equal on both gallic and tannic acid media. 14 fungi. (Fig. 1, *A* and *B*.)

Group 2.—Growth on gallic acid medium, good, mat diameter much greater than on tannic acid medium. Five fungi. (Fig. 1, *C* and *D*.)

Group 3.—Good growth on gallic acid medium, none or only a trace on tannic acid medium. 17 fungi. (Fig. 1, *E* and *F*.)

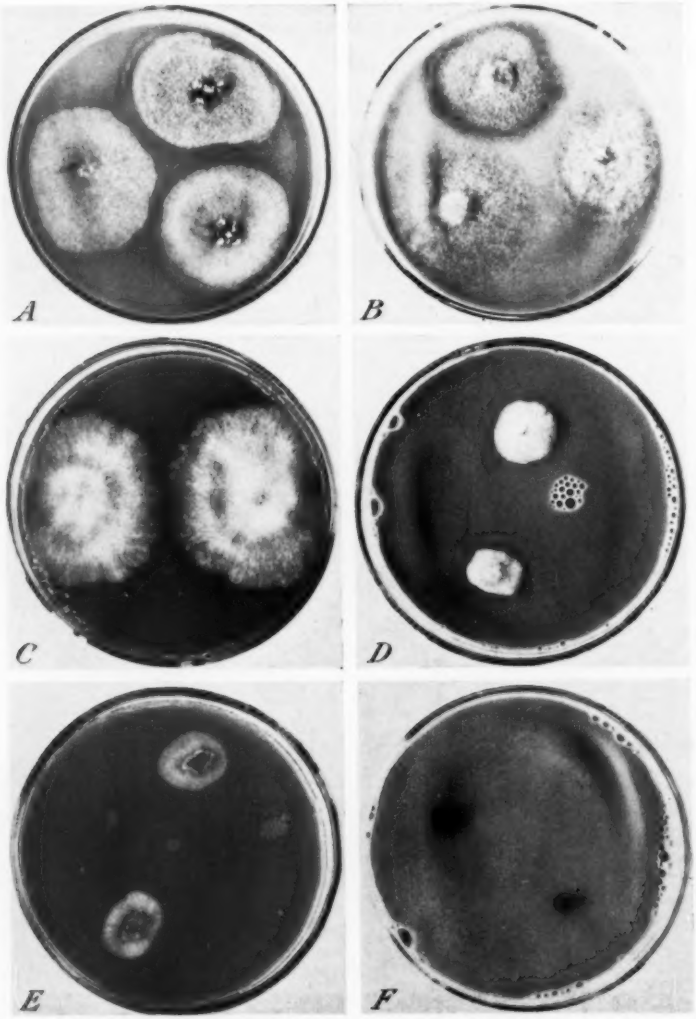

FIGURE 1.—Negative or nonreacting groups. *A* and *B*, Group 1 as illustrated by *Polyporus sulphureus;* mat diameters for a given species approximately the same on gallic (*A*) and tannic (*B*) acid media. *C* and *D*, Group 2, as illustrated by *Coniophora suffocata;* good growth on gallic (*C*) acid medium, poor on tannic (*D*) acid medium. *E* and *F*, Group 3 as illustrated by *Daedalea juniperina;* good growth on gallic (*E*) acid medium, no growth or only a trace on tannic (*F*) acid medium.

POSITIVE-REACTING FUNGI

Group 4.—No growth, or only a trace, on both gallic and tannic acid media. 34 fungi. (Fig. 2, *A* and *B*.)

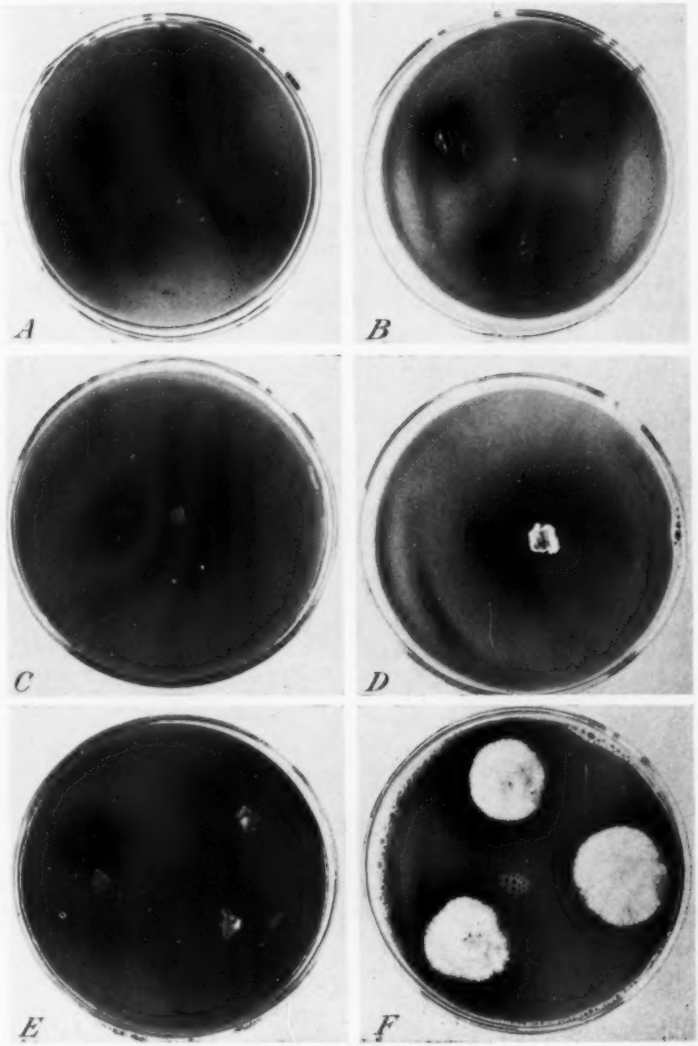

FIGURE 2.—Positive-reacting groups. *A* and *B*, Group 4 as illustrated by *Polyporus glomeratus;* no growth or only a trace on gallic (*A*) and tannic (*B*) acid media. *C* and *D*, Group 5 as illustrated by *Stereum murrayi;* no growth or only a trace on gallic (*C*) acid medium, trace to 25 mm on tannic (*D*) acid medium. *E* and *F*, Group 6 as illustrated by *Irpex mollis;* no growth or only a trace on gallic (*E*) acid medium, growth 25–50 mm on tannic (*F*) acid medium.

Group 5.—No growth or only a trace on gallic acid medium, mat diameter 25 mm or less on tannic acid. 57 fungi, all strong reactors. (Fig. 2, *C* and *D*.)

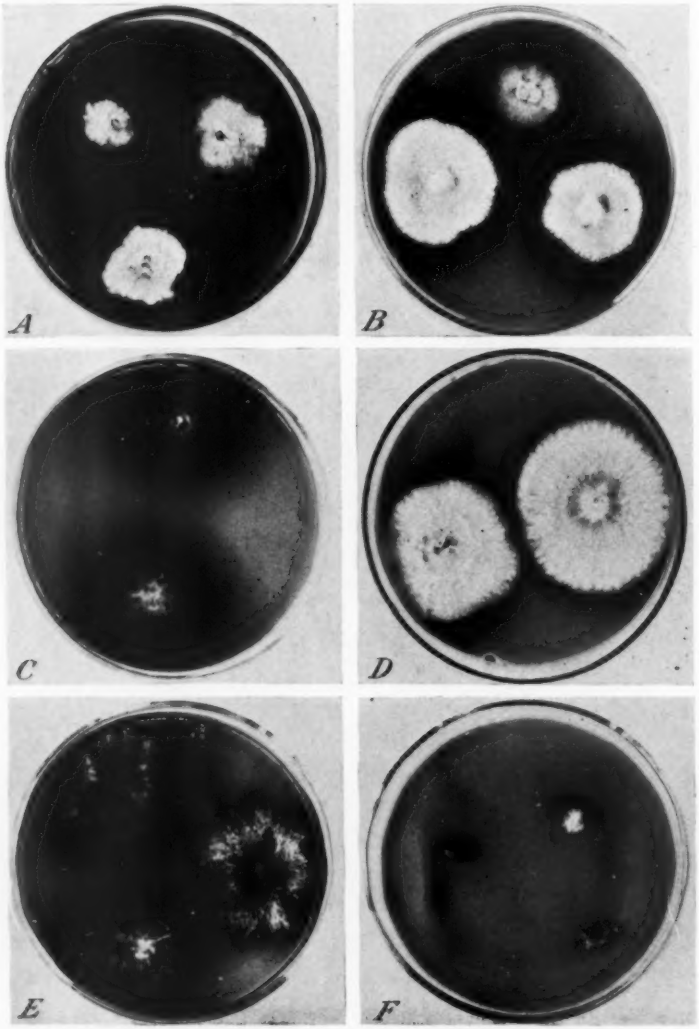

FIGURE 3.—Positive-reacting groups. *A* and *B*, Group 7 as illustrated by *Polyporus compactus;* mat diameters approximately equal on both gallic (*A*) and tannic (*B*) acid media. *C* and *D*, Group 8 as illustrated by *Polyporus distortus;* slight growth on gallis (*C*) acid medium, good growth on tannic (*D*) acid medium. *E* and *F*, Group 9 as illustrated by *Polyporus tulipiferus;* good growth on gallic (*E*) acid medium, none to a trace on tannic (*F*) acid medium.

Group 6.—Growth none or only a trace on gallic acid medium, growth 25–50 mm on tannic acid. 22 fungi. (Fig. 2, *E* and *F*.)

Group 7.—Mat diameters about equal on both media. 31 fungi. (Fig. 3, *A* and *B*.)

Group 8.—Fair growth on gallic acid medium, good growth on tannic acid medium. 12 fungi. (Fig. 3, *C* and *D*.)

Group 9.—Good growth on gallic acid medium, none to trace on tannic acid medium, often weak reactors, some requiring 14 days for definite results. Nine fungi. (Fig. 3, *E* and *F*.)

FUNGI HAVING NEGATIVE OR POSITIVE REACTION, DEPENDING ON MEDIUM

Group 10.—Reaction negative on gallic acid medium, positive on tannic acid medium, with good growth on both. One fungus (*Schizophyllum commune*).

DISCUSSION

Naturally, since arbitrary limits have been prescribed for the above-mentioned groups, certain fungi should be expected to show growth behavior which may fall within two or more groups. Each fungus is placed in a group on the basis of its most consistent and characteristic growth behavior, and the relative consistency with which different isolates fall into a given group may vary for the different species. For this reason, in comparing growth groups of two different species, more significance should be attached to growth reactions involving widely separated groups such as groups 4 and 9 than to those involving closely related groups such as 4 and 5 or 5 and 6. This applies to both positive and negative reactors. In order to demonstrate the consistency with which a given fungus would fall into a definite group on repeated trials of the same isolate, or with a number of isolates, the data for a few fungi, for which several isolates were available, have been analyzed. Ninety-five percent of the recorded conclusive trials place *Polyporus compactus* in group 7 and 5 percent in group 6 (21 trials); 54 percent place *P. cuticularis* in group 5 and 46 percent in group 4 (22 trials); 73 percent place *P. dryophilus* in group 7, 21 percent in group 9, and 5 percent in group 4 (19 trials); 82 percent place *P. hispidus* in group 5 and 17 percent in group 4 (17 trials); 97 percent place *P. spraguei* in group 1 and 3 percent in group 3 (33 trials); 73 percent place *P. supinus* in group 5 and 27 percent in group 6 (15 trials); and 72 percent place *Poria andersonii* in group 5 and 28 percent in group 4 (18 trials).

The foregoing growth and reaction groups are constant and distinct enough to be useful in the identification of certain fungi. For example, cultures of *Polyporus pargamenus* and *P. tulipiferus* are sufficiently similar to be confused in the laboratory. On 0.5-percent gallic and tannic acid media, however, the reaction of the former falls under group 5, that of the latter under group 9. The differences between the two species on these media cannot be confused. In many cases identification of isolations from unnamed sporophores may be assured if the type of decay associated with the sporophore can be determined. The oxidase test is useful in such cases as well as in the general determination of decay types for fungi taken from incipient decay before the typical stage manifests itself. *P. schweinitzii* and *P. circinatus* sporophores are similar, but cultures of the two fungi can be readily separated on gallic and tannic acid media, as the former is a nonreactor while the latter is a positive reactor.

A number of other instances where the oxidase test has been of value could be cited, but the few listed here give a general idea of cases in which the oxidase reaction, correlated with other information, has been of particular value.

A column showing type of decay is included in table 1 to give some idea of the kind of rot that is usually associated with each fungus. The type of rot has not been determined by pure-culture methods but by the association of sporophores with the rots in question. In many cases, as in certain groups of the Thelephoraceae, where no definite association of rot and fungus has been found, the space is left blank.

As used in the paper, the term "brown rot" refers only to the brown cubical type of decay. Most of the others are classified as white rots, although many are somewhat colored owing to the color of the wood or to changes associated with incipient stages of infection. For instance, *Stereum frustulosum*, *S. subpileatum*, and *Hydnum erinaceus* are usually present in brown-stained wood, but the wood is white or light-colored in the final stages of decay.

Of the 210 fungi that were tested for the presence of oxidases 36 were negative, i. e., they did not form brown diffusion zones on either medium; 8 were inconsistent, i. e., they were usually negative, but certain isolations gave slight brown diffusion zones on one or both media; 165 were positive; and 1 (*Schizophyllum commune*) gave consistently negative results with gallic acid medium but positive results with tannic acid, with good growth on both.

Of the 36 fungi that were consistently negative, 29 are associated with brown carbonizing rots;[7] the type of decay for 4 fungi could not be checked. *Fistulina hepatica* causes a darkening of the wood but not a typical brown rot; *Stereum frustulosum* causes a white pocket rot, which produces dark discoloring of the wood about the decayed pockets; and *Polyporus dichrous* is associated with what appears to be a definite white rot. Of the eight fungi giving inconsistent, but mainly negative results, seven are definitely brown rot fungi while *S. subpileatum* is associated with a white pocket rot with darkening of the surrounding wood.

Of the 165 fungi giving consistently positive oxidase reactions, 156 are associated with white rot;[8] the type of decay was unknown for 9 species. *Schizophyllum commune*, associated with a white decay, was negative on gallic acid but positive on tannic acid medium, with good growth on both.

Eighty percent of the negative reactors were correctly diagnosed as brown rot fungi and 96 percent of the positive reactors were cor-

[7] The majority of brown rot fungi showed no signs of reaction on either gallic or tannic acid medium. This was especially true of those species producing white mycelia in culture. The species that gave inconsistent results usually formed yellow, buff, or brown mycelia in culture, and it was often difficult to decide whether the faint discoloration, as seen from the under side of the Petri dish, was due to the color of the mat or to reaction with the medium. This was especially true on the transparent gallic acid medium.

[8] White rot fungi showed considerable variation in the intensity of the brown diffusion zone and in the time required for its formation. Very strong reactors gave a definite zone in 1 or 2 days. The great majority produced definite results in 7 days while only a few fungi required a longer time for the zone to form. Strongest reactors gave positive results with little or no growth, especially on gallic acid. A few gave results only with growing mycelium. For this reason tests showing no reaction and no growth on either medium were considered inconclusive, but tests showing a reaction without growth were considered conclusive. Since all fungi do not react equally well with both tannic and gallic acid media both should be used when the purpose of the test is to determine decay type by the oxidase reaction.

rectly diagnosed as white rot fungi. Thus Bavendamm's generalization, that brown rot fungi are negative when tested for oxidases by the use of gallic and tannic acids and that white rot fungi give positive reactions with the same media is essentially correct.

SUMMARY

Two hundred and ten fungi were tested for oxidases, using Bavendamm's gallic and tannic acid method.

Twenty-nine, or 80 percent, of the fungi associated with brown carbonizing rots gave no reaction with gallic or tannic acid media. One (*Fistulina hepatica*), associated with a dark firm rot, was negative, as was *Stereum frustulosum*, associated with a white pocket rot, and *Polyporus dichrous*, associated with a white rot. Four whose rots were unknown gave no reaction. Seven fungi associated with brown carbonizing rots gave inconsistent reactions.

One hundred and fifty-six, or 96 percent, of those fungi associated with white rots gave positive reactions. Nine specimens whose rots were unknown also gave positive reactions. *Stereum subpileatum*, associated with a white pocket rot similar to *S. frustulosum*, gave mostly positive results on gallic acid but was negative on tannic acid. *Schizophyllum commune*, a white rot fungus, was negative on gallic acid medium but positive on tannic acid medium, with good growth on both media.

With white rot fungi the intensity of reaction and the time required for the reaction to take place showed considerable variation.

Growth behavior for different species on gallic acid and tannic acid media showed variations of value in the cultural identification of fungi. Ten different growth and reaction groups are described and the species are listed under these groups.

AN ANALYSIS OF THE BLOOD OF THE SIXTH-INSTAR SOUTHERN ARMYWORM (PRODENIA ERIDANIA) [1]

By FRANK H. BABERS

Associate biochemist, Bureau of Entomology and Plant Quarantine, United States Department of Agriculture

INTRODUCTION

The body cavity of insects contains only one tissue fluid, the so-called blood, or hemolymph. The composition of this blood varies greatly from species to species, and there is also marked variation in individuals of the same species. It becomes important, therefore, to determine the approximate concentration of the various constituents in the blood of insects that are being used in experiments on insect physiology. Mature (sixth-instar) larvae of the southern armyworm (*Prodenia eridania* (Cram.)) have been used in large numbers in these laboratories for such experiments. In order that a perfusion fluid similar to the normal blood might be prepared, a quantitative analysis of the blood of this insect was made. It was also necessary to know the approximate normal concentrations of the various constituents of the blood before attempting a study of the effect of feeding various foodstuffs and certain toxic substances on the processes of digestion and absorption. The results of the several analyses are recorded in this report.

Most of the analytical procedures were developed for use with human blood, and, with few exceptions, they were found entirely satisfactory for use with insect blood. Many of the methods are recorded by Peters and Van Slyke (*28*),[2] and the modifications of the original procedures that these authors recommend have been followed.

EXPERIMENTAL DATA

GENERAL REACTIONS

The blood from mature larvae of the southern armyworm is a viscous fluid ranging in color from bright green to a dirty, nondescript yellow. It begins to clot almost immediately after being drawn from the insect, the clotting apparently being of the type classified by Yeager and Knight (*37*) as cell coagulation.

The blood from the southern armyworm does not cause clotting when added to solutions of fibrinogen or thrombin obtained from normal horse serum.

The volume of blood varies considerably among individuals and seems to be dependent on both the age of the larvae and the type of food on which it has fed. The quantity of blood obtained from one insect was usually from 0.07 to 0.2 ml, with an average of 0.12 ml.

[1] Received for publication May 27, 1938; issued November 1938.

[2] Italic numbers in parentheses refer to Literature Cited, p. 704.

On exposure to air the blood darkens rapidly from the surface down, and is soon almost black. This darkening is probably due to the presence of tyrosinase, since a suspension of tyrosine turns black in a few hours after a small quantity of insect blood has been added.

Both clotting and darkening are prevented and the insect is killed by immersion in water at 60° C. for 1 minute. There is no visible coagulation of plasma[3] proteins from this treatment.

The Millon, Hopkins-Cole, xanthoproteic, Adamkiewicz, biuret, and Molisch tests were all strongly positive, as was the sulphuric acid-petroleum ether test for carotinoids. The Pettenkofer test for bile salts was also positive. The ether extract of a sample of blood boiled with an equal volume of 12 N hydrochloric acid and a pinch of naphthoresorcin (Tollens' reagents) was a bright reddish purple. This color was entirely different from the color given by glucuronic or galacturonic acid. The sodium nitroprusside and bromine tests were negative.

DIGESTIVE ENZYMES IN THE BLOOD

Invertase and lipase were demonstrated in whole blood by using 1 percent of sucrose and cream as the respective substrates. Amylase was not found, nor were the proteolytic enzymes pepsin, trypsin, and erepsin. In man proteolytic enzymes, together with their antibodies, are found in serum. These antibodies render the enzymes inactive and prevent digestion of the blood proteins. Extraction of the blood with toluene, chloroform, or certain other organic solvents either removes these antienzymes or renders them inactive. Even after extraction with toluene and chloroform, no proteolytic enzymes were demonstrated in the blood of the southern armyworm.

HYDROGEN-ION CONCENTRATION

The pH value of the blood was determined electrometrically with the micro quinhydrone electrode described by Cullen (*15*). Blood was obtained by snipping off a leg while holding the insect under mineral oil. At 24.5° C. the pH value of the blood from six larvae varied between 6.40 and 6.67, with an average of 6.53.

OSMOTIC PRESSURE

The osmotic pressure of whole freshly drawn blood was determined by the freezing-point method, with use of a copper-constantan thermocouple. Δ was found to be $-0.84°$ C. This corresponds to an osmotic pressure of 10.12 atmospheres, based on a molecular freezing-point lowering of 1.86° and an osmotic pressure of 22.4 atmospheres.

BODY TEMPERATURE

Incidental to the determination of the freezing point of the blood, the body temperature of the insect was determined by inserting a copper-constantan thermocouple in the anus of insects wired down on cork. When the room temperature was 23.8° C., the average temperature of six insects after equilibrium was reached was 22.3°, or 1.5° below room temperature.

[3] In all cases where the term "plasma" is used, reference is to the fluid obtained after centrifugation of blood from such heat-treated insects, whereas the term "serum" refers to the fluid obtained by centrifugation of blood that clotted after being drawn from living insects.

DENSITY

Blood from larvae killed by heating at 60° C. for 45 seconds was weighed in a micropycnometer at 25° and the weight compared with that of the same volume of distilled water at 25°. The density was 1.032 g per cubic centimeter.

OXYGEN CAPACITY

Blood from live larvae was collected under mineral oil to retard the darkening that begins soon after the blood is drawn from the insect and exposed to air. Blood so drawn remained undarkened for at least 2 hours. After standing for 15 minutes in the ice box, the tube was centrifuged and the clear serum pipetted off. One-milliliter samples were analyzed for oxygen capacity in the Van Slyke manometric apparatus by both the regular Van Slyke method and the following modification: The sample was washed into the gas chamber with 2.5 ml of 0.9-percent sodium chloride solution and equilibrated with air by shaking for 2 minutes. A drop of caprylic alcohol was added, followed by 0.2 ml of 5-percent sodium cyanide solution, and the dissolved gases were released by evacuating for 2 minutes. The carbon dioxide was absorbed with 1 ml of normal sodium hydroxide solution and the manometer read. One milliliter of alkaline pyrogallic solution (1 volume of 22-percent pyrogallic acid plus 4 volumes of 10-percent potassium hydroxide) was then added and the manometer again read after 2 minutes had been allowed for drainage and absorption. The difference in the two readings multiplied by Van Slyke's factors gives the volume-percent of oxygen gas in the sample.

Determinations were also made with whole blood. It was difficult to obtain homogeneous samples of whole blood because of the rapid clotting, but the clot was broken up as much as possible before the sample was taken. Clotting was so retarded that homogeneous samples were obtained when the blood was allowed to drop directly from the insect into an equal volume of saturated sodium oxalate solution. Blanks in which a physiological salt solution was substituted for the insect blood were run with each determination. In every experiment the oxygen capacity of the insect blood and serum was slightly less than that of normal salt solution.

As a check on the methods of analysis, the oxygen capacity of the serum from lobster (*Homarus americanus*) was determined. This blood is known to contain the oxygen carrier hemocyanin in appreciable quantities.

The results of these determinations are shown in table 1.

TABLE 1.—*Oxygen content of the blood of the southern armyworm*

Manner of liberating gases	Material tested	Temperature	Oxygen capacity		Oxygen unaccounted for by blank
			Material	Blank	
		°C.	*Volume-percent*	*Volume-percent*	*Volume-percent*
Van Slyke technique	Blood-saturated sodium oxalate (1:1)	24	2.39	2.48	----------
	Whole blood	25	2.45	2.53	----------
	Serum	26	1.88	2.02	----------
Sodium cyanide	Whole blood	15	1.64	1.73	----------
	Serum	24	1.81	1.98	----------
	Lobster blood	21	2.96	2.03	0.93

CHEMICAL ANALYSES

Nonprotein nitrogen was determined on the filtrate obtained by treating plasma with nitrogen-free trichloroacetic acid and centrifuging. The micro-Kjeldahl method, with potassium persulphate and copper sulphate as catalysts, was used. The total plasma nitrogen was determined by micro-Kjeldahl analysis. The protein content was obtained by subtracting the amount of nitrogen in the protein-free filtrate and multiplying the difference by the factor 6.25. The general nature of these proteins has not yet been determined.

The micro-Van Slyke method was used for determining amino nitrogen in the protein-free filtrate obtained on treating plasma with sodium tungstate reagent.

The apparent "creatinine" in the blood was determined by the method of Folin and Wu (*18*).

The blood was analyzed for urea by the xanthydrol colorimetric method of Beattie (*2*).

The uric acid in the Folin and Wu protein-free filtrate of serum was determined colorimetrically by Benedict's direct method (*3*, *4*).

The colorimetric method of Shohl and Bennett (*29*) was used for the determination of potassium. Attempts to use cobaltinitrite methods were unsuccessful, as checks could not be obtained.

Blood was analyzed for sodium by the Butler and Tuthill (*12*) modification of the method of Barber and Kolthoff.

Calcium was determined by the modified Halverson and Bergeim method described by Peters and Van Slyke (*28*, *p. 767*).

Magnesium in the filtrate from the calcium determination described above was precipitated as magnesium ammonium phosphate (*11*), and the phosphate was determined by Tisdall's colorimetric method (*32*).

A measured volume of pooled blood from a number of insects that had been killed and their blood fixed by immersion in water at 60° C. for 1 minute was added to 10 volumes of 10-percent trichloroacetic acid solution in a 15-cc centrifuge tube. After standing for 10 minutes, the tubes were centrifuged and the supernatant liquid was poured quantitatively into a 100-ml Kjeldahl flask. The precipitate was dissolved in a small volume of normal sodium hydroxide solution and rinsed into another Kjeldahl flask. Both solutions were boiled down to small volume, ashed, and the copper sulphide was precipitated by the method of Ansbacher, Remington, and Culp (*1*). The copper sulphide was dissolved in concentrated nitric acid, the acid removed by evaporation on the steam bath, and the copper determined colorimetrically by the method of Callan and Henderson (*13*) with the use of sodium diethyl dithiocarbamate.

Inorganic sulphur in the blood was determined as barium sulphate by the gravimetric procedure of Loeb and Benedict (*21*). When 15 ml of the protein-free filtrate was hydrolyzed with 4 ml of normal hydrochloric acid for 20 minutes, the barium sulphate precipitated gave inorganic plus ethereal sulphur. Nonprotein sulphur was determined on 10 ml of the protein-free filtrate after hydrolysis with the zinc nitrate oxidizing mixture described by Denis and Reed (*16*).

Chlorides were determined on 0.2-ml samples of whole blood by Patterson's method (*27*), after digestion by the open Carius method. There were 119.8 mg of chlorine per 100 ml of blood, which corresponds to 197.5 mg of sodium chloride.

Total reducing substances were found by the Somogyi micro modification of the Shaffer-Hartmann method (*30*). This method proved more satisfactory than the Hagedorn-Jensen procedure. Fermentable substances were determined by treatment with yeast according to the procedure described by Peters and Van Slyke (*28, p. 479*).

The colorimetric method of Mendel and Goldscheider (*25*) was used to determine lactic acid. Only a trace was found by this procedure.

Pooled blood from insects killed by immersion in water at 60° C. for 1 minute was analyzed for glycogen by the method of Creveld (*14*), the only modification being that the glucose liberated was determined with the micro Shaffer-Hartmann-Somogyi reagent I (*30*). Glycogen was obtained from the glucose value by multiplying by the factor 0.927.

Total lipoids (lipids), fatty acids, and cholesterol were determined by the chromate oxidation method as described by Bloor (*7*). The phospholipoids were isolated and determined according to Bloor's procedure (*8*). The direct extraction and colorimetric method of Leiboff (*20*) and the method of Bloor, Pelken, and Allen (*9*) were used in the determination of cholesterol. Samples corresponding to 2 ml of blood were used in each case. Some trouble was experienced with the colorimetric method because of the formation of a yellowish color which was extremely difficult to compare with the standard. This was overcome by washing the chloroform extract with water and drying with calcium chloride before developing the color. The use of a red filter in the colorimeter was also of benefit.

The total phosphorus in whole blood was determined by Tisdall's (*32*) colorimetric method after digestion with a mixture of nitric and sulphuric acids. Inorganic phosphorus was determined by the same method after removal of the proteins with trichloroacetic acid.

The amounts of the various substances found in 100 ml of blood are as follows:

	Milligrams
Nonprotein nitrogen	401. 4
Total nitrogen	568. 4
Amino nitrogen	235. 2
Proteins (protein nitrogen×6.25)	1, 043. 8
"Creatinine" (apparent)	8. 0
Urea	6. 2
Uric acid	14. 8
Potassium	155. 0
Sodium	51. 2
Calcium	36. 75
Magnesium	17. 2
Copper, total	4. 93
Copper, in protein-free filtrate	2. 94
Copper, in protein precipitate	1. 99
Sulphur, inorganic	30. 7
Sulphur, ethereal	0. 4
Sulphur, total nonprotein	44. 4
Chlorine	119. 8
Chlorine (as sodium chloride)	197. 5
Total reducing substances (as glucose)	65. 9
Reducing substances fermented by yeast (as glucose)	11. 1
Reducing substances not fermented by yeast (as glucose)	54. 8
Lactic acid	Trace
Glycogen	3. 29
Lipoids, total	320. 5
Phospholipoids	99. 0
Cholesterol	12. 8
Phosphorus, total	123. 3
Phosphorus, inorganic	17. 59

DISCUSSION

In a paper that has been widely quoted, Muttkowski (*26*) reported the presence of copper and oxygen in the blood of the various insects he tested, but he gave no quantitative results. He found also that the bloods gave positive tests with benzidine, *o*-tolidine, and guaiac. He failed to find copper in human blood. From these experiments he drew the sweeping conclusions that all insect bloods contain a respiratory protein, and that this respiratory protein is hemocyanin, the copper-containing oxygen carrier found in the blood of many marine invertebrates.

The almost universal occurrence of copper is well established. Its presence in plants, human blood and tissue, and the blood of many other animals has been demonstrated by McHargue (*22*, *23*), Warburg (*34*), Elvehjem, Steenbock, and Hart (*17*), and others, and so its mere presence in insect blood would seem to be without special significance. Melvin (*24*) has shown that certain insects accumulate copper, and that the cast skins of others have more copper than the insects producing them. The fact that copper was found in such relatively large quantities by Melvin and also by the author in the insects studied may very well mean that copper has a definite physiological function in insects, but at the present time there seems to be no real evidence for stating just what this function may be.

The qualitative presence of oxygen in insect blood as reported by Muttkowski is, of course, valueless as evidence either for or against the presence of a respiratory protein, since there is no qualitative method of distinguishing between dissolved oxygen and oxygen bound by any of the known respiratory proteins. Oxygen capacities for insects and other invertebrates of approximately the same order of magnitude as those for mammals were reported by Griffiths (*19*). Much of his work, however, has been refuted by Winterstein (*36*), and in the light of later evidence his values must be considered far too high.

Bishop (*5*) found no evidence of a respiratory protein and found also that the quantity of oxygen present in the blood of the honeybee larva was not appreciably more than would be physically dissolved. Bishop used the regular Van Slyke apparatus and technique.

In the present work the writer has obtained similar results with the blood of the southern armyworm larva. It is well known that oxyhemocyanin is not reduced by ferricyanide as is hemoglobin. Oxyhemocyanin is reduced by cyanides, however, and this fact has been utilized by many investigators in determining the oxygen capacity of bloods known to contain hemocyanin. For this reason, although there is no evidence that insect blood contains a hemocyanin, a sodium cyanide solution, as well as the regular Van Slyke reagent, was used during the course of the analyses. The oxygen obtained from southern armyworm blood was in all cases slightly less than was obtained from equal volumes of physiological saline.

In tests with benzidine, *o*-tolidine, and guaiac, the writer obtained positive results only with benzidine, and then only when a saturated solution was used. Little or no significance can be attached to these tests, since positive reactions are given by raw milk, pus, saliva, many plant extracts, potatoes, and other substances in addition to the respiratory proteins. Diastases, as well as the so-called oxidation enzymes

so common in plant fungi, seeds, and bacteria, also give positive reactions. Ferric and cupric salts react positively, as do many other inorganic materials.

These experiments seem to indicate that, if hemocyanin or any other respiratory protein is present in southern armyworm blood, it occurs in such small quantities as to play no part in the normal respiration of the insect.

Although Muttkowski concluded that hemocyanin is present in insect blood, he states: "No doubt some of this reaction [the test for oxygen in the blood] was due to oxygen dissolved in the blood serum, although the serum has no power to combine with oxygen like a respiratory protein." This conclusion, if supported by experimental evidence, should have prevented him from suggesting the presence of hemocyanin in insect blood, since serum is the only portion of blood from which a hemocyanin has been isolated. As far as the author knows, it has never been demonstrated in cells.

The writer's value for the oxygen capacity of lobster blood is similar to that reported by Stedman and Stedman (*31*) in their work on hemocyanins from various sources.

At the present time no explanation is made for any seeming abnormalities in the composition of the blood. The ratio of amino nitrogen to nonprotein nitrogen is extremely high in comparison with this ratio in vertebrates, but the total nitrogen and protein nitrogen seem low. The urea and uric acid content of the blood is high, but this is to be expected, since it has been shown that insects excrete most of their waste nitrogen in the form of uric acid (*35*).

Magnesium is present in high concentration, but this also seems to be generally true with insect blood. Inorganic sulphur is high, but reducing substances, especially substances fermentable by yeast, are extremely low. This probably accounts for the fact that only a trace of lactic acid was found. The composition of the nonfermentable portion is not known, but the reduction is probably due in part to uric acid. The high concentration of glycogen in the blood may also explain the low glucose content to some extent.

Voit (*33*) has shown that glycogen can be formed in mammals from various monosaccharides and disaccharides. Of the substances from which glycogen is usually formed, carbohydrates are probably the most common in the normal diet of the southern armyworm. The insect stores large quantities of glycogen in its fat body for use during pupation. It is probable, therefore, that the insect possesses an efficient glycogen-manufacturing mechanism by which carbohydrates are transformed into glycogen. This in turn may account for the relatively small quantities of free reducing sugars normally present in the blood.

Both total phosphorus and inorganic phosphorus are very high as compared with the phosphorus in human serum, but the values are not unusual for insects. Brecher (*10*) found phosphorus to be high in *Pieris brassicae;* and Bishop, Briggs, and Ronzoni (*6*) found a similar condition in the blood of honeybee larvae.

Cholesterol is such an important constituent of human blood that the relatively small quantities found in the larval blood seem especially interesting.

SUMMARY

The blood (hemolymph) of sixth-instar larvae of the southern armyworm (*Prodenia eridania* (Cram.)) has been analyzed.

A brief description has been given of the blood with some of its physical properties, viz, approximate blood volume, color, density, osmotic pressure, and pH value.

The blood gives positive Millon, Hopkins-Cole, xanthoproteic, Adamkiewicz, biuret, Molisch, Pettenkofer, and carotinoid reactions. The sodium nitroprusside and bromine tests were negative.

Some of the digestive enzymes of the blood have been determined. Invertase and lipase were demonstrated, but amylase, pepsin, trypsin, and erepsin were not found.

The body temperature of the insect was found to be 1.5° C. below room temperature.

The oxygen capacity was found to be slightly less than that of normal saline, and so it was concluded that no respiratory protein or pigment was present.

Total nitrogen, nonprotein nitrogen, amino nitrogen, proteins, apparent "creatinine," urea, uric acid, potassium, sodium, calcium, magnesium, copper, total, inorganic, and ethereal sulphur, chlorides, total reducing substances, substances fermented by yeast, glycogen, total lipoids, phospholipoids, cholesterol, and total and inorganic phosphorus have been determined quantitatively.

No evidence was obtained to account for the seeming abnormal concentrations of several of the constituents.

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THE BIOLOGICAL DISPOSITION OF ROTENONE AFTER INGESTION BY THE SOUTHERN ARMYWORM [1]

By P. A. WOKE

Assistant entomologist, Division of Control Investigations, Bureau of Entomology and Plant Quarantine, United States Department of Agriculture

INTRODUCTION

The toxic action of ingested rotenone has been observed to vary markedly with different species of insects. Campbell [2] in 1929 (*8*) [3], using the sandwich method described by Campbell and Filmer (*2*), determined the median lethal dose for fourth-instar silkworms (*Bombyx mori L.*) to be near 0.003 mg per gram of body weight. Hansberry and Richardson (*4*) state that this value was twice duplicated in their laboratory, and that the same value had been obtained by Kagy. They also report a median lethal dose for *Vanessa cardui* (L.) of 0.03 mg per gram. Swingle (*9*) reports that cabbage worms died within 24 hours after eating very small quantities of a gelatin film containing derris. There are indications [2] that rotenone may be toxic in similar small dosages to several other species of insects. There are also indications [2] that ingested rotenone exerts little or no toxic action on certain other species. Hansberry and Richardson (*4*) found that a dose of 0.49 mg per gram was apparently nontoxic to *Heliothis obsoleta* (F.). This dose, they point out, is 16 times the median lethal dose for *Vanessa cardui* and 160 times that for *Bombyx mori*. The high specificity of this toxicant suggests a relationship to differences in the physiological characteristics of the different species of insects and to their food (*8*).

According to Lightbody and Mathews (*7*), the toxicity of rotenone to white rats is increased by feeding it dissolved in oil, or by feeding oil following rotenone.

Ambrose and Haag (*1*) report that their studies on the toxicity of derris to mammals indicate that part or all of the rotenone ingested may be absorbed and slowly excreted into the intestinal tract, and that it is eliminated in the feces either free or in some form closely resembling rotenone. Species difference in toxic action was observed.

The present investigation was undertaken to determine the biological disposition of rotenone after ingestion by the southern armyworm (*Prodenia eridania* (Cram.)).

Preliminary experiments had shown that sixth-instar larvae of the southern armyworm ingest turnip-leaf sandwiches containing 5mg of rotenone as readily as they do similar sandwiches without rotenone, and that healthy larvae apparently suffer no ill effects from the ingestion of this quantity of rotenone. There was some evidence, however, that toxic action may be produced under certain, perhaps abnormal, conditions.

[1] Received for publication May 19, 1938; issued November 1938.

[2] CAMPBELL, F. L. REVIEW OF INFORMATION ON THE INSECTICIDAL VALUE OF ROTENONE. U. S. Bur. Ent. Mimeo. Cir. E-298, 28 pp. 1932.

[3] Italic numbers in parentheses refer to Literature Cited, p. 712.

MATERIALS AND METHODS

The larvae used in these experiments were sixth instars of the southern armyworm reared in a greenhouse insectary on turnip plants and cut lettuce. The rotenone used was as nearly pure as could be determined by chemical examination.[4]

Toxicity was determined by the use of mosquito larvae (*Culex fatigans* Wied.) as biological indicators according to the method reported by Campbell and associates (*3*, *5*). This method has been used successfully for the determination of comparative toxicity of a number of insecticidal substances, including rotenone (*5*), to which mosquito larvae are rather sensitive.

The rotenone was fed to the armyworm larvae in sandwiches, each of which was prepared as follows: Disks 22 mm in diameter were cut from turnip leaves. One disk was lightly coated on the smooth side with starch paste, and 5 mg of rotenone was distributed evenly over the surface. A second disk, also lightly coated with starch paste on the smooth side, was caused to adhere to the first disk with the rotenone between.

The larvae were removed from food several hours before being offered the sandwiches. Each larva was placed in a 150-ml beaker with one sandwich and kept for 18 hours overnight. In the morning all the larvae that had ingested entire sandwiches were selected and were conserved on fresh turnip leaves 24 hours longer. It was assumed that this period of time would be ample for the complete evacuation from the alimentary canal of all the rotenone or rotenone derivatives that might be eliminated.

The presence of unaltered rotenone in the tissues, in the contents of the gut, or in the feces of the larvae would be detectable by testing acetone extracts of these materials against mosquito larvae used as biological indicators. Accordingly the materials were prepared for this purpose. All the feces egested by all the armyworm larvae during the 42-hour period were collected and combined. The armyworm larvae were dissected, and the contents of the guts of all the larvae were removed and combined. The tissues of all the larvae were also combined. Each of the three materials was ground with sand, desiccated at 50° C. in a partial vacuum, reground, and extracted four times at room temperature with 50 ml of acetone. Satisfactory extraction was obtained by this method, as indicated by the application to the marc of the color test for rotenone (*6*).

By experiment it was determined that 0.2 ml of acetone per milligram of rotenone is well above the minimum quantity required to hold the rotenone in solution, and that this quantity of acetone, without rotenone, in 0.1 percent gelatin solution is not toxic to mosquito larvae in the lowest dilution employed, as described below. It was also determined that, if impurities were present in the acetone, they were not toxic to mosquito larvae even when concentrated by the evaporation of a large quantity of acetone. On the basis of these facts, the excess acetone was eliminated by evaporation at 25°–30° C. in a partial vacuum, leaving the final volume of acetone as 0.2 ml for each milligram of rotenone originally ingested by the larvae.

[4] A sample of the rotenone was analyzed for purity by C. C. Cassil, of the Division of Insecticide Investigations of this Bureau. The Gross-Smith color test showed 98 percent purity, and the optical rotatory power, $[\alpha]_{D_t}^{20^\circ}$ was found to be −222. 5°, as compared with −220.8° given by pure rotenone.

Each of the three extracts that were prepared as outlined above was tested against mosquito larvae to determine the presence of a substance toxic to these insects. The absence of such toxic substance would indicate the absence of rotenone, while the presence of such would require its identification as rotenone or some other toxic substance, probably a rotenone derivative. Each of the three extracts was placed in distilled water containing 0.1 percent of gelatin. On the assumption, which proved correct, that all the rotenone ingested by the larvae might be in one extract, dilutions of the extracts at the rate of 1 part of rotenone to 100,000, 500,000, 750,000, and 1,000,000 parts of gelatin solution were prepared, One hundred milliliters of each of the four dilutions of each of the three extracts (12 samples) was placed in each of 12 Erlenmeyer flasks. Twelve lots of 100 mosquito larvae each were prepared, and one lot was placed in each flask. The mortality was determined after exposure for 20 hours at 29° C. in the dark.

The tests, which were prepared as outlined above, were accompanied by three checks. The term "test" as here used refers to the diluted extracts of material prepared from southern armyworm larvae that had ingested rotenone. One check consisted of diluted extracts of materials prepared from armyworm larvae that had not ingested rotenone and to which rotenone had not been added. The purpose of this check was to determine the possible toxic influence of the extracts of the larval materials alone on the mosquito larvae. The second check was like the first except that a quantity of rotenone equal to that ingested by the larvae from which the tests were prepared was mixed with each of the three materials—feces, contents of the gut, and tissues of the larvae—at the time the materials were ground before extraction. The purpose of this check was to determine the toxic influence of a known quantity of rotenone added to the larval materials. A third check consisted of dilutions of a gelatin solution of a quantity of rotenone equal to that ingested by the armyworm larvae from which the tests were prepared. Its purpose was to determine the toxic action of a known quantity of rotenone without the larval materials. In all respects except as indicated the checks were prepared in precisely the same manner as were the tests.

The arrangement as outlined above constituted one trial, and was carried out three times to increase the reliability of the results. Essentially the same results were obtained in all three trials.

From 7 to 10 armyworm larvae were used for the preparation of the test materials in each trial, or a minimum of 24 larvae for the three trials. In each trial the same numbers of larvae were used in the preparation of each check that were prepared from armyworm materials. A minimum of 72 larvae were therefore used altogether in the preparation of the tests and two checks.

RESULTS

The results are summarized in table 1. Each figure is the average mortality of the 1,200 mosquito larvae in all four dilutions and in all three trials.

From an examination of these figures it is apparent that the extracts of the tissues and of the gut contents of rotenone-fed larvae possessed practically no toxicity, while the extract of the feces of the same

larvae were highly toxic. The possible influence of the extracts of the larval materials alone on the toxicity may be disregarded, since the negligible mortality in the no-rotenone check indicates that they possess little or no toxicity. The mortality in the second check is high and indicates approximately the mortality that might be expected should all the rotenone ingested by the armyworm larvae appear in any one of the three test materials. Rotenone in gelatin solution without larval extracts gave similar high mortality.

TABLE 1.—*Toxicity to mosquito larvae of acetone extracts of tissues, gut contents, and feces of southern armyworm larvae that had ingested rotenone, and of similar extracts of larvae that had not ingested rotenone when rotenone was and was not added before extraction*

Acetone extracts of materials from armyworm larvae	Average mortality of mosquito larvae exposed to—		
	Extracts of armyworm larvae that had ingested rotenone (tests)	Extracts of armyworm larvae that had not ingested rotenone (checks)	
		No rotenone added before extraction	Rotenone added before extraction
	Percent	*Percent*	*Percent*
Larval tissues (gut contents removed)	0.75	0.33	86.00
Gut contents	.91	.17	85.25
Feces	82.50	1.17	85.41
Gelatin solution without larval extract (check)			89.00

The practical absence of mortality of mosquito larvae exposed to extracts from tissues and gut contents of larvae that had ingested rotenone indicates that rotenone is not present in the tissues of larvae or in the contents of the digestive tract 24 to 42 hours after ingestion of the substance, at least in quantities sufficient to be detected by the method used.

The toxicity of the fecal extracts of rotenone-fed larvae was similar to the toxicity of the fecal extract of the rotenone-added check and also of the rotenone-only check, and indicates the presence of rotenone or of some other toxic agent.

Microscopic examination of the feces of larvae that had ingested rotenone revealed the presence of quantities of apparently unaltered particles, assumed to be those of rotenone by their similarity to the same material before ingestion and by their absence from feces of larvae that had not ingested rotenone. The occurrence of toxicity in feces of larvae that have ingested rotenone equivalent to that in feces of normal larvae to which a quantity of rotenone equal to that ingested by the first group of larvae has been added probably indicates, if no chemical change has occurred, that rotenone ingested by these larvae is wholly or largely eliminated in the feces.

Since the known derivatives of rotenone that are at all likely to be producible in the gut of the insect are nontoxic or of decidedly lower toxicity than rotenone, and since the color test for rotenone (*6*) was observed, it is probable that chemical change has not occurred and that the observed toxicity is due to the presence of rotenone.

Preliminary experiments in which the desiccation of the larval

material, the extractions, and the evaporation of the acetone were performed with heat at atmospheric pressure gave similar results. The reduced-pressure method finally used, however, prevents the destruction of the rotenone or reduction of its toxicity due to heat or to prolonged boiling in solution.

SUPPLEMENTARY EXPERIMENTS

DISPOSITION OF INGESTED ROTENONE AS DETERMINED BY CHEMICAL METHODS

To substantiate the biological evidence with chemical evidence, feces from 11 larvae that had ingested 5 mg of rotenone each were tested [5] for the possible identification of rotenone and determination of the quantity that passed through the insect unchanged. By the Gross-Smith colorimetric method of estimating rotenone, 45 mg of rotenone was found in the entire feces and 2 mg was found in the acetone washings of the beakers in which the feces had been collected. This is a total of 47 mg out of the 55 mg that had been fed to the 11 larvae. That the material in the feces was really rotenone was determined by the fact that it gave the Durham blue color test, and more definitely by the fact that crystals obtained from the extract had the common habit of rotenone crystals and showed certain optical properties, that is, parallel extinction and alpha and gamma indices of refraction, the same as pure rotenone.

EFFECTS OF ISOLATED LARVAL TISSUES ON THE TOXICITY OF ROTENONE

Another experiment was conducted to determine whether the toxicity of rotenone would be affected by incubation with any of the separate isolated tissues or the gut contents of the southern armyworm. The blood, skin, tissues of the digestive tract and the malpighian tubules, muscular tissues, fat body, contents of the foregut and the hindgut, and contents of the midgut were isolated from 10 healthy larvae and corresponding kinds of materials combined. Five milligrams of rotenone was mixed with each kind of material, which was then ground with sand in a mortar. The thoroughly mixed and ground materials were incubated in the dark for 18 hours at 29° C., desiccated, extracted with acetone, and tested against mosquito larvae for the presence of toxic principles as previously described. Corresponding materials from 10 other healthy armyworm larvae were treated in the same manner, except that rotenone was not mixed with them, to serve as checks for determination of the possible toxic effects of the larval material alone on the mosquito larvae. An acetone solution of rotenone served to demonstrate the toxic effects of rotenone alone.

The mosquito larvae appeared to be unaffected by the extracts of the larval tissue and the contents of the digestive tract that had been incubated without rotenone, as indicated by the low mortality, which averaged only 0.83 percent. The mortality of the mosquito larvae in the tests in which rotenone was incubated with the various tissues and with the contents of the digestive tract averaged 98 percent, corresponding closely to that of the rotenone-only check, which averaged 99.3 percent. The results of this experiment indicate that little

[5] These analyses were made by C. C. Cassil and E. L. Gooden, of the Division of Insecticide Investigations.

or no reduction in toxicity of rotenone occurs during incubation for 18 hours in the dark at 29° C. with any of the tissues or with the gut contents.

SUMMARY AND CONCLUSIONS

Experiments were made to determine the biological disposition of rotenone after ingestion by the southern armyworm (*Prodenia eridania* (Cram.)). Sixth-instar larvae that had been reared on turnip plants and cut lettuce were used. Rotenone was fed to the larvae in sandwiches, and after intervals of time acetone extracts of the tissues, gut contents, and feces were prepared and tested against mosquito larvae for the determination of toxicity. Suitable checks were employed.

The results show that the southern armyworm larva, after ingesting 5 mg of rotenone, eliminates all or most of the substance with its feces. This result was substantiated by chemical tests.

It was also shown that the toxicity of finely powdered rotenone to mosquito larvae is not altered to a demonstrable extent after 18 hours' incubation in the dark at 29° C. with the various tissues or with the contents of the digestive tract.

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